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Unique and Overlapping Actions of Type I and III IFNs in Influenza A Virus Infection and Implications for Therapy

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Abstract

Influenza A virus (IAV) poses a significant public health burden. Severe disease is characterised by infected lung airway epithelial cells (AECs), inflammation and tissue damage. However, disease severity differs between individuals and this cannot be entirely explained by inter-individual differences in pre-existing immunity or comorbidities. Host-specific, genetically determined factors must contribute to susceptibility. Type I interferon ($\text{IFN}\alpha\beta$) is known to have antiviral function in vitro, but its role in restricting IAV infection in vivo is controversial. We demonstrate that responsiveness to $\text{IFN}\alpha\beta$ signalling is a host-specific determinant with protective or pathogenic potential which determines the severity of IAV-induced disease. IAV infected 129, CBA/J and DBA mouse strains showed dramatically increased mortality and lung damage, yet higher levels of pulmonary $\text{IFN}\alpha\beta$, compared to C57BL/6 or BALB/C mice. Ablation of $\text{IFN}\alpha\beta\text{R}$ signalling in 129 mice markedly reduced mortality, levels of proinflammatory cytokines, inflammatory cell recruitment and AEC apoptosis. Susceptibility to IAV-induced disease is influenced by the number of functional alleles for the $\text{IFN}\alpha\beta\text{R}$ subunit, IFNAR1 , within the genome. $\text{IFNAR1}^{+/-}$ (129) mice were less susceptible than wild type 129s yet more so than $\text{IFN}\alpha\beta\text{R}^{-/-}$ (129) mice. Conversely, triplication of a section of murine chromosome 16 that includes IFNAR1 in C57BL/6 mice enhanced $\text{IFN}\alpha$ response to IAV infection and downstream immunopathology. Finally, $\text{IFN}\alpha$ therapy of infected B6.A2G-Mx1 mice reduced IAV titers, yet increased secretion of proinflammatory cytokines, innate cell recruitment and AEC apoptosis in the lung, due to the potent immunostimulatory capability to $\text{IFN}\alpha$. In

contrast, treatment with IFN λ , whose receptor is largely restricted to AECs, promoted IAV control without exacerbating IAV-induced inflammation. Thus, by manipulating the IFN $\alpha\beta$ signal in various ways, we demonstrate that excessive IFN $\alpha\beta$ in IAV infection can increase AEC death and enhance proinflammatory responses that ultimately increase disease severity. Our findings have important implications for prediction and treatment of severe influenza.

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Abbreviations

129	129SvEv or 129S7
Ab	Antibody
ADCC	Ab-dependent cell-mediated cytotoxicity
AEC	airway epithelial cell
ALI	Air-liquid interface
AM	Alveolar macrophage
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
B6	C57BL/6
BAL	Bronchioalveolar lavage
BM	Bone Marrow
BMDM	Bone Marrow derived Macrophages
Cal09	A/California/04/09
CARD	Caspase Associated Recruitment Domain
CC	Collaborative Cross
CCR	C-C chemokine receptor type 2
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary Deoxyribonucleic acid
CLP	Common lymphoid progenitors
cRNA	Complementary RNA
CSF	Colony-stimulating factor
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine
DC	Dendritic cell
delNS1 IAV	delta NS1influenza A virus
DISC	Death-inducing signalling complex
DS	Down's Syndrome
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FasL	Fas ligand
Flt3	Fms-like tyrosine kinase 3
G	Guanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association studies

H&E	hematoxylin and eosin
HA	hemagglutinin
HAT	human airway trypsin-like protease
HCV	hepatitis C virus
HLA	Human leucocyte antigen
HSV2	herpes simplex virus 2
i.n	intranasal
i.p.	intraperitoneal
IAE	influenza-associated encephalopathy
IAV	Influenza A virus
IFITM3	interferon-inducible transmembrane protein 3
IFN	interferon
IFN $\alpha\beta$	type I interferon
IFN $\alpha\beta$ R	type I interferon receptor
IFN γ	type II interferon
IFN λ	type III interferon
IFN λ R	type III interferon receptor
Ig	immunoglobulin
IL	interleukin
IL-1a	interleukin -1R antagonist
IMc	inflammatory monocyte
iNOS	inducible nitric oxide synthase
IP-10	IFN γ -induced protein 10
IPA	Ingenuity pathway analysis
IRAK1	IL-1 receptor-associated kinase 1
IRF	interferon regulatory factor
ISG	IFN stimulated gene
ISGF3	Interferon-stimulated gene factor 3
JAK1	Janus activated kinas 1
KC	Keratinocyte-Derived Chemokine
LCMV	Lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
Ly6C	lymphocyte antigen 6C
M1	matrix protein 1
M2	matrix protein 2
mAb	monoclonal antibody
MAVs	Mitochondrial antiviral-signalling protein (MAVS)
MCP-1	monocyte chemotactic protein 1
MDA-5	Melanoma Differentiation-Associated protein 5
MDCK	Madin-Darby Canine Kidney
MEF	Mouse embryonic fibroblast
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MHV	Mouse hepatitis virus
MIG	Monokine induced by IFN γ
Mip-1 α	Macrophage inflammatory protein 1 α
Mip-1 β	Macrophage Inflammatory Protein-1 β
Mip-2	Macrophage Inflammatory Protein-1 β
Mip-3 α	Macrophage inflammatory protein 1 α
Mx	Orthomyxovirus resistance gene
MyD88	Myeloid differentiation primary response gene 88
NA	Neuraminidase
NBF	Neutral buffered formaldehyde
NEP	Nuclear export protein
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural Killer cell
NLRP3	NOD-LRR- and pyrin domain containing 3
NLS	Nuclear localization signals
NOS2	Nitric oxide synthase 2
NP	Nucleoprotein
NS1	Non-structural protein
OAS	2'-5'oligoadenylate
PAMPs	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-L1	Programmed death-ligand 1
pDC	plasmacytoid dendritic cell
PGE2	Prostaglandin E2
PKR	Protein kinase R
PR8	A/Puerto Rico/8/34
PRR	Pattern recognition receptor
PVM	Pneumovirus for mice
qPCR	quantitative real-time PCR
QTL	Quantitative trait locus
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complexes
Rsad2	Radical S-Adenosyl Methionine Domain Containing 2
S-OIV	Swine-origin influenza virus
SIg	Surface Immunoglobulin
SNP	Single nucleotide polymorphism
ssRNA	single stranded RNA
STAT	Signal transducer and activator of transcription
T regs	Regulatory T cells

TCID ₅₀	50% tissue culture infective dose
TIR	Toll/IL-1R homologous region
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine S1 member 2
TNF	Tumour necrosis factor
TRAF6	TNF receptor- associated factor 6
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
TRIM25	Tripartite Motif Containing 25
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TYK2	Tyrosine kinase 2
U	Uracil
WHO	World Health Organisation
Wt	Wild type
X31	H3N2 A/Hong Kong/X31/68
Dp8Tyb	Dp(16Ifnar1-Runx1)8TybEmcf/B6
129	129SvEv or 129S7
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Mip-1 β	Macrophage Inflammatory Protein-1 β
Mip-2	Macrophage Inflammatory Protein-1 β
Mip-3 α	Macrophage inflammatory protein 1 α
Mx	Orthomyxovirus resistance gene
MyD88	Myeloid differentiation primary response gene 88
NA	Neuraminidase
NBF	Neutral buffered formaldehyde
NEP	Nuclear export protein
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
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TRIM25	Tripartite Motif Containing 25
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TYK2	Tyrosine kinase 2
U	Uracil
WHO	World Health Organisation
Wt	Wild type
X31	H3N2 A/Hong Kong/X31/68

Chapter 1. Introduction

The immune system is amazing. It is inherent to all living things, from the simplest single cell organisms to higher order mammals such as humans; all life forms on this planet have some version of an immune system. Host immunity is vital to an organism's survival and propagation as, aside from injury, infection is the primary cause of death prior to an organism reaching sexual maturity. This infinitely complex system has evolved over hundreds of millions of years to recognise and eliminate or control foreign or dangerous material from the body. The immune system is tasked with combating invading pathogens such as viruses, bacteria, fungi and parasites as well as removing aberrant or damaged host cells.

In mammals, the immune system can be divided into two parts: the innate and the adaptive. Adaptive immunity is characterised by the development or augmentation of host defence mechanisms to a specific stimulus. Cells of the adaptive immune system, specifically T and B cells, express antigen specific receptors that are generated through a random process known as somatic recombination, and specificities that recognise pathogens or aberrant self are enriched during an immune response by a process called clonal selection and expansion. Specific antigen recognition not only eliminates the invading pathogen and facilitates recovery from disease, but also leaves the host with immune memory, thereby allowing for faster resolution upon reinfection.

As important as the adaptive immune response is, it relies on innate immunity for activation and modulation and for holding pathogens in check until the rare pathogen specific T and B cells of the adaptive immune response have expanded enough to control and eliminate the pathogen. Inborn or innate immunity consists of physical, chemical and biological barriers (e.g.: skin, mucosal surfaces and gut microflora), the complement system which 'complements' cellular

function, and a cornucopia of innate immune cell types. Cells of the innate immune system are vital to host defence. Not only do they limit the spread of an invading pathogen or aberrant cells (e.g.: cancer) through generic defence mechanisms such as macrophage or neutrophil phagocytosis or killing by natural killer (NK) cells, they also secrete cytokines which orchestrate both adaptive and innate immune cell recruitment and function, and they acquire and process antigen for presentation to the adaptive immune system. Furthermore, cytokines secreted during the innate immune response alert host cells to the presence of pathogens and thereby induce upregulation of intracellular defence mechanisms to prevent pathogen invasion.

It is difficult to communicate the immense complexity of mammalian immunity. To a varied extent both innate and adaptive immune responses are tailored to a specific threat, whether that be an invading pathogen, cancerous cells or sterile damage. It will take more than my lifetime to grasp all aspects of the host immune systems, so unsurprisingly, this thesis focuses on the host immune response to one pathogen, Influenza A virus (IAV), and how differences in the immune response due to host genetic background alters disease outcome. Particularly, how the antiviral cytokine families type I interferon ($\text{IFN}\alpha\beta$) and type III interferon ($\text{IFN}\lambda$) and the innate inflammatory cell types plasmacytoid dendritic cells (pDCs) and inflammatory monocytes (IMcs) contribute to inflammation, resolution of infection or immunopathology.

1.1 Influenza A Virus

IAV was first isolated by Smith, Andrews, and Laidlaw in 1933 (Smith, 1933). IAV, along with influenza B and C virus (IBV and ICV), Isavirus, Thogotovirus and Quaranjavirus make up the Orthomyxoviridae family. All Influenza species have a common ancestry but are now too genetically divergent to exchange genetic material with one another. Within the family, IAV infection in humans is most common, although IBV has also been shown to cause seasonal outbreaks (Julkunen et al., 2000). IAV is a major cause of upper and lower respiratory tract infections in humans, indeed the World Health Organisation (WHO) estimates influenza epidemics cause three to five million cases of severe illness and about 250,000 to 500,000 deaths worldwide annually (Stohr, 2002).

IAV is further subtyped into strains based on expression of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes have been characterised, but seasonal influenza outbreaks are currently caused only by IAV strains H1N1 and H3N2 and IBV (Kreijtz et al., 2011). In addition to the public health burden caused by seasonal IAV epidemics, this virus also poses the threat of pandemics. 1918 saw the introduction of IAV strain H1N1 into the human population, and this resulted in the most dramatic IAV pandemic to date. Termed 'Spanish flu', this H1N1 strain infected 500 million people and caused up to 50 million fatalities, making it one of the deadliest natural disasters in human history (Kash et al., 2006). H1N1 IAV circulated in humans until 1957, when the 'Asian Flu' outbreak occurred and H2N2 IAV completely replaced the H1N1 subtype as the circulating strain. Approximately a decade later, H3N2 superseded H2N2 and caused the 1968 'Hong Kong' pandemic. Around the mid 1970s IAV H1N1 strains reappeared without causing a major pandemic (Kreijtz et

al., 2011). That is, until swine-origin H1N1 (H1N1 S-OIV) emerged in 2009, triggering the first pandemic of the 21st century and resulting in over 200,000 deaths. Fatalities associated with seasonal epidemics are normally restricted to the very old, very young or already immunocompromised (Poehling et al., 2006), yet the mortality burden of H1N1 S-OIV fell most heavily on otherwise healthy adults (Simonsen et al., 2013).

Although comparatively less effective at spreading through humans, other IAV subtypes also represent a serious threat to human health. H5N1, H7N7 and H9N2 are sporadically transmitted from animals to humans (de Wit et al., 2008). Avian H5N1 in particular, causes severe disease in humans with over 560 human cases being recorded since 2003, of which 60% proved to be fatal (Kreijtz et al., 2011). Avian IAV infection is characterised by an exceptionally severe host inflammatory response (Peiris et al., 2004).

The IAV genome is comprised of eight negative sense single stranded ribonucleic acid (RNA, ssRNA) segments, which normally code for 11 viral gene products. These segments are coated with nucleoprotein (NP) and the trimeric viral RNA polymerase (consisting of PB1, PB2 and PA proteins), collectively forming ribonucleoprotein (RNP) complexes. Matrix protein 1 (M1) encases the RNP complexes, which in turn is enveloped by a lipid layer containing HA and NA. Finally, the ion channel matrix protein 2 (M2) transverses the lipid envelope (Bouvier and Palese, 2008; Palese, 2004) (Figure 1A). Notably, IAV does not possess RNA proofreading enzymes, with the RNP making an error roughly every 10 thousand nucleotides, approximately the length of IAV genome. Thus, many of the newly generated influenza virions are mutants. This poor genomic fidelity allows for what is known as antigenic drift, i.e: a gradual change in the viral

Figure 1

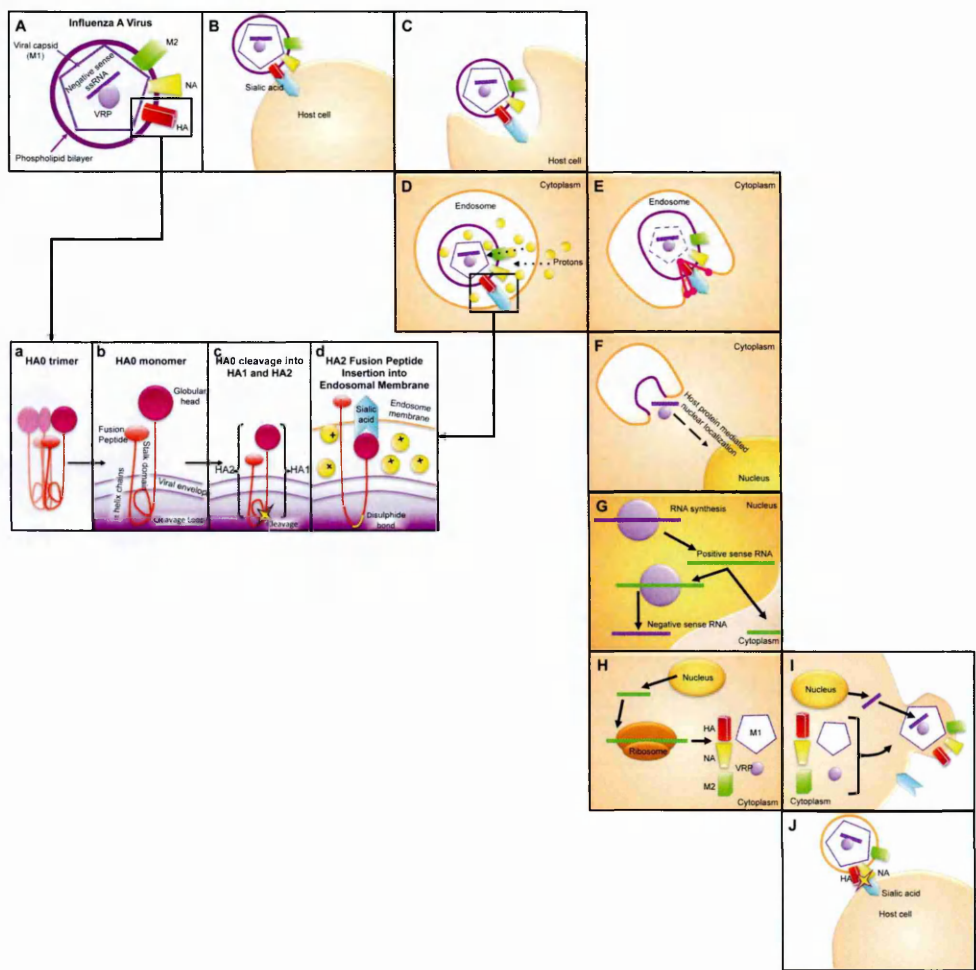


Figure 1: Features of IAV HA and IAV replication. (A) IAV virion, surface proteins Hemagglutinin (HA), Neuraminidase (NA) and Matrix protein 2 (M2) shown in red, yellow and green, respectively are drawn only once in IAV viral envelope (purple circle) for simplicity. The viral capsid is made up of Matrix protein 1 (M1) (purple pentagon) and encases the IAV genome. IAV genome is made up of eight negative sense ssRNA segments (simplified to purple line) and is coated with nucleoprotein (NP) (not shown) and the trimeric viral RNA polymerase (VRP). (a-c) HA is found as a homotrimer, each HA is made up of a globular head that contains the receptor binding domain, a fusion peptide, a cleavage loop and a stalk domain. HA0 is matured by host protease mediated cleavage of the cleavage loop resulting in two subunits: HA1 and HA2 which remain complexed together via a disulphide bond. (B) IAV virion attaches to host cell through binding of HA to silica acid. (C) IAV virion is endocytosed by host cell and enters the cytoplasm in an endosome. (D and d) Protons are pumped into the endosome and the drop in pH within the endosome triggers a conformational change in HA and exposes the HA2 fusion peptide. The M2 ion channel is opened and acidification of the viral core occurs. (E) Fusion of the viral and endosomal membranes. (F) Release of IAV genome and associated proteins into the host cell's cytoplasm. (G) VRP initiates RNA synthesis on viral RNA, generating a positive sense copy which serves as a template for the IAV negative sense ssRNA and (H) produces capped mRNAs for viral protein production by the host cell ribosome. (I) New IAV virions are assembled at the cell surface and bud off, using the host cell membrane as a new viral envelope (orange circle). (J) NA cleaves HA and sialic acid binding to allow for virion detachment.

antigens over time. Furthermore, as the IAV genome is segmented, IAV subtypes can reassort between one another. When two different viruses co-infect a single host, the reassortment of genetic segments within the host “mixing vessel” may occur, effectively resulting in massive alterations in IAV protein antigens, in particular surface glycoproteins, a phenomenon called antigenic shift. It is through antigenic drift and shift that new strains of IAV are able to circumvent recognition by host adaptive immunity in organisms that have been previously exposed to IAV. Effectively, antigenic shift has the capacity to generate entirely novel IAV strains which may have pandemic potential (Webster et al., 1992). Host immunity to a specific strain of IAV is easily acquired, however due to this capacity for rapid evolution, IAV is able to cause epidemics on almost a yearly basis. Cross protection between IAV strains by the adaptive immune system occurs, however this is directly dependent upon the similarity between virus subtypes. Indeed, it is hypothesised that the higher mortality rate of H1N1 S-OIV infected individuals aged less than 65 years was due to the novelty of H1N1 S-OIV compared to circulating IAV strains of the previous five decades (Hancock et al., 2009; Skountzou et al., 2010).

The IAV surface protein HA is vital to the establishment of IAV infection. Once inhaled into the human respiratory tract, IAV targets airway epithelial cells by viral recognition of sialic acid moieties on the host cell surface (Figure 1B). Different HA molecules have different affinities for α -2,3 or α -3,6 linkages between the terminal sialic acid and galactose. Importantly, distribution of these linkages strongly impacts on IAV tropism and host specificity. As human respiratory epithelia primarily express α 2,6-linked sialic acid, human IAV HAs have binding preference to this, whereas avian influenza viruses have a preference for α 2,3-

linked sialic acid (Bouvier and Palese, 2008; Julkunen et al., 2000; Leung et al., 2012).

HA is a homotrimer that forms spikes on the viral lipid membrane (Figure 1a). The HA precursor HA0, matures by cleavage mediated by host-encoded proteases into two subunits which remain complexed together via a disulphide bond. Of the subunits HA1 contains the receptor binding domain and HA2 comprises the fusion peptide (Wilson et al., 1981) (Figure 1b-c). Upon HA spike binding to sialic acid, IAV virion endocytosis is mediated by clathrin-mediated and clathrin-independent mechanisms, and virus enters the host cell in an endosome (Figure 1C). The low pH within the endosome triggers a massive conformational change in HA and exposing the HA2 fusion peptide (Figure 1D, 1d). This fusion peptide consequently induces the fusion of the viral and endosomal membranes (Bouvier and Palese, 2008) (Figure 1E). The acidic environment within the endosome also opens the M2 ion channels in the IAV envelope (Holsinger and Lamb, 1991; Pinto et al., 1992). Opening of this proton-selective ion channel pumps hydrogen ions into the viral core, and this acidification of the core releases IAV RNP from M1 into the host cell's cytoplasm (Pinto and Lamb, 2006) (Figure 1D-F).

IAV RNP localises to the host cell nucleus through host proteins responding to the nuclear localization signals (NLSs) provided by the proteins that make up RNP (NP, PB1, PB2 and PA) (Samji, 2009) (Figure 1F). Although IAV genome replication is not entirely understood, it is known that this occurs independent of a primer; instead, the IAV RNA polymerase complex initiates RNA synthesis internally on viral RNA, generating a positive sense (complementary) copy of the IAV genome which serves as a template for the influenza negative sense ssRNA and

producing capped mRNAs for viral protein production (Figure 1G). Once generated, IAV positive sense ssRNA export from the nucleus is mediated by the association of M1 and IAV's nuclear export protein (NEP). Virus proteins are then synthesised in the cytoplasm and packaged into new IAV virions, which are assembled at the cell surface (Figure 1H-I). Finally, NA acts as a sialidase, cleaving the surface moieties on the host cell to allow for virion detachment and therefore infection propagation (Bouvier and Palese, 2008; Doherty et al., 2006; Julkunen et al., 2000).

Productive replication of IAV is generally restricted to airway epithelial cells (AECs). This is due to the requirement of HA0 to be cleaved by a host cell protease into the subunits HA1 and HA2 to gain its fusion capacity (Figure 1a-c). This is hypothesised to occur on the plasma membrane, either during attachment and entry into the cell or during assembly and budding of progeny virus. Low pathogenicity strains of IAV carry a HA cleavage site with a monobasic motif susceptible to trypsin-like proteases such as HAT (human airway trypsin-like protease) and TMPRSS2 (transmembrane protease serine S1 member 2), and the tissue distribution of these proteases is restricted to AECs (Bottcher-Friebertshauser et al., 2010). However, highly pathogenic avian IAV strains have been shown to possess a polybasic HA cleavage site cleavable by furin (Stieneke-Grober et al., 1992), which is ubiquitous and therefore supports systemic viral replication. Some strains of highly pathogenic IAV may therefore achieve productive infection in immune cells such as macrophages.

1.2 Natural and Experimental hosts of IAV

Along with humans, IAV has a broad range of host species, including pigs, horses, wild mammals, and birds. Wild waterfowl are considered the natural reservoir of IAVs, as the prevalence of IAV is high in this species and many combinations of HA and NA have been identified in wild waterfowl (Olsen et al., 2006). As pigs are particularly susceptible to infection with both human and avian IAV strains, this animal can serve as a mixing vessel for IAV strain (Ma et al., 2009). This is of particular concern as transmission from swine to humans is not rare (Robinson et al., 2007), and therefore the emergence of a radically novel IAV strain of avian origin with the potential to easily infect and transmit between humans may occur.

A number of animal models have been employed to study IAV infection in vivo. These include nonhuman primates, swine, domestic poultry, guinea pigs, cats, and dogs, however the most common experimental models are mice and to a lesser extent ferrets. As mentioned, human IAV strains bind to sialic acids attached to galactose in an α -2,6 configuration, and a distinct advantage of the ferret model is that ferrets have a similar distribution of α -2,6-linkage sialic acid receptors within their respiratory tract. This is consequently thought to be responsible for ferret susceptibility to human IAV strains (Jayaraman et al., 2012) and for virus transmission between ferrets. It has also been noted that both the avian H5N1 and human H3N2 influenza viruses exhibit similar patterns of virus attachment to tissues from both species (van Riel et al., 2006; van Riel et al., 2007). Ferrets recapitulate many of the clinical signs observed in human IAV infection, including fever, nasal congestion and discharge, anorexia, sneezing, and lethargy (Bouvier and Lowen, 2010). Sneezing is of particular importance as it promotes IAV

transmission. Given the limited availability of reagents for detailed phenotyping, the lack of genetically modified strains and the ferret's exquisite sneeze reflex, this model is primarily used to study viral and host factors responsible for transmission of IAV and effectiveness of anti-IAV drugs, and historically for serological studies (Francis and Magill, 1935; Govorkova et al., 2007; Herlocher et al., 2001; Maines et al., 2006; Mendel et al., 1998). Yet, even though humans and ferrets share similar distribution of sialic acid receptors, IAV infection in ferrets rarely progresses to the lower respiratory tract to induce pneumonia, even with high pathogenic strains such as avian IAV or 1918 H1N1 (Maher and DeStefano, 2004). This is of importance as α 2,3-linkages (i.e.: sialic acid receptor configuration preferred for avian IAV strain binding) become more prevalent in the lower respiratory tract of humans (Bouvier and Palese, 2008; Julkunen et al., 2000; Leung et al., 2012), and therefore the ferret may not be an appropriate model for IAV induced pneumonia and disease course of highly pathogenic avian IAV strains.

In contrast to ferrets, the mouse is the most commonly used experimental model of IAV infection. This is an incredibly convenient model, the multitude of genetically mutant murine strains greatly facilitates investigation into proteins and pathways involved in IAV protection or disease pathogenesis and to determine the mechanism of action of proposed therapies. Furthermore, whole genome analysis techniques coupled with the range of genetically distinct inbred strains has allowed for comparisons of the host immune response to IAV on different host genetic backgrounds. In particular the international Collaborative Cross (CC) project, which uses a multiparental recombinant inbred panel generated from eight founder inbred laboratory and wild-derived mouse strains, has allowed for

identification of quantitative trait loci (QTL) that modulate in IAV disease course, however this is a relatively recent advancement (Ferris et al., 2013; Threadgill and Churchill, 2012).

Clinical symptoms of IAV induced disease in mice are characterised by huddling, ruffled fur, lethargy, anorexia, weight loss and laboured breathing, and in contrast to humans, mice develop hypothermia (Yang and Evans, 1961). IAV infection of mice does replicate many of the cardinal features of IAV induced disease in humans, including: proinflammatory cytokine secretion, interstitial inflammatory cell recruitment, lung edema, and haemorrhage (Kash et al., 2006; Radigan et al., 2012). However, the mouse is not a natural host for influenza viruses and many human IAV strains must be adapted by passage through murine tissue in order for them to replicate efficiently within the murine respiratory tract. An important contributor of murine resistance to human IAV strains is the sialic acid moieties of the murine respiratory tract are of the α -2,3 linkage to galactose (Radigan et al., 2015). Yet, this may be an advantage as α -2,3-linkage sialic acid receptors become more prevalent in the lower respiratory tract of humans and therefore mice may be an appropriate model for viral pneumonia. Indeed, IAV replication and tissue damage typically occur within the lower respiratory tract of the mouse. Although sialic acid moieties are important for IAV infectivity, other factors must play a role as some IAV strains have not required adaptation for infection in mice. Unsurprisingly, this includes many avian strains, but also human IAVs such as the 1918 H1N1 and the 2009 S-OIV strains (Radigan et al., 2015). Furthermore, there is evidence of transmission between mice of select IAV strains (Edenborough et al., 2012).

The appropriately named orthomyxovirus resistance gene family (Mx family) is another factor which likely contributes to the mouse not being a natural host for IAV. Mx proteins are GTPases and induced by IFN $\alpha\beta$ and IFN λ . Murine Mx1 inhibits IAV replication by interrupting IAV PB2-NP interaction. Mx1 is a potent restrictor of IAV both in vitro and in vivo and indeed, mice carrying a functional Mx1 locus are exquisitely resistant to IAV infection. However, most inbred laboratory mouse strains have a deletion of three exons or a nonsense mutation in the Mx1 locus making the protein non-functional, and therefore, inbred laboratory mice are significantly more susceptible to IAV infection (Horisberger et al., 1983; Lindenmann, 1962; Lindenmann et al., 1963; Salomon et al., 2007; Verhelst et al., 2012). This is significant as natural IAV host species including humans, have a homologue of the Mx1 gene (MxA in humans), and therefore, congenic mouse strains which express functional Mx1 have been generated. Yet, whether or not this is a better model for human infection of IAV is arguable, as Mx homologs in natural IAV hosts such as chickens or humans are comparatively less effective at specifically inhibiting IAV and do not restrict IAV replication in the same manner (Pavlovic et al., 1992). It is not unreasonable to question the use of a system where the action of a single gene so entirely overpowers all others, to model a comparatively weaker effect in humans. Furthermore, some IAV strains have found ways to evade Mx antiviral action (Pavlovic et al., 1995; Pavlovic et al., 1992; Schusser et al., 2011).

No model can fully reproduce influenza infection, however the myriad of tools and the ease of use of murine studies make this animal model a vital component to IAV research. Indeed, all experiments in this thesis and the majority of the studies discussed and referenced in this thesis employ mouse models of IAV

infection, in both an Mx function and Mx non-functional context, and use of primary murine cells in vitro.

1.3 Resolution of IAV infection

Once infection is established, clearance of IAV from a host is absolutely dependent upon adaptive immunity. The adaptive immune response can be separated into cellular (or T cell mediated) and humoral (B cell) immunity, both of which launch IAV specific responses to IAV. In an otherwise normal context, either B cell produced neutralizing antibodies or the cytotoxic CD8⁺ T cell response alone is sufficient to control and resolve primary IAV infection and later homologous challenge. Both responses also impart varying degrees of protection to secondary infection with heterologous IAV strains (Eichelberger et al., 1991a; Epstein et al., 1998; Scherle et al., 1992; Topham et al., 1996).

The adaptive immune response to IAV is orchestrated by the innate. IAV peptides are presented to adaptive immune cells by antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells themselves. Conventional DCs (cDCs) are considered to be the most effective cell type for IAV presentation and priming of naïve T cells. cDCs reside between the airway epithelia and the basal membrane and extend dendrites through the tight junctions between epithelial cells to monitor the airway lumen. cDCs can acquire IAV protein via direct IAV infection or by phagocytosis of opsonised virions or apoptotic bodies from other infected cells. Upon APC infection, IAV antigens are digested by the proteasome into peptides, while IAV antigen taken up by the APC is processed into peptides in the endosomes by proteolysis. IAV peptides are subsequently bound to

major histocompatibility complex (MHC) molecules in either the endoplasmatic reticulum (MHC class I) or endosomes or lysosomes (MHC class II) for presentation on the cell surface. cDCs migrate to the lung draining lymph nodes (mediastinal) and spleen within the first 36 hours post infection to present antigen to T cells in order to induce clonal expansion and maturation of antigen specific clones. B cells are also able to present antigen through capture of antigen via surface immunoglobulin (SIg). This process is highly selective as only B cells expressing the appropriate SIg will take up the IAV antigen (Hamilton-Easton and Eichelberger, 1995; Janeway, 1999).

Antibody (Ab) subtypes that make up the IAV humoral response are primarily immunoglobulin (Ig) M, IgA and IgG. IgM initiates complement-mediated neutralization of influenza virus, and presence of this isotype is a hallmark of primary infection (Fernandez Gonzalez et al., 2008; Jayasekera et al., 2007). IgA antibodies are produced locally at the epithelial layer and transported across epithelia and along the mucus to help protect epithelial cells from infection. IgA has also been shown to neutralize IAV intracellularly (Mazanec et al., 1995). Finally, long-lived Ab-mediated protection from IAV is achieved by the induction of IgG antibodies (Clements and Murphy, 1986; Koutsonanos et al., 2011; Onodera et al., 2012).

B cell-produced antibodies which correlate with protective immunity to IAV are commonly specific for the IAV surface glycoprotein HA (Gerhard, 2001). HA specific antibodies inhibit virus attachment and entry in host cells by binding to HA's trimeric globular head (Figure 1a-c). HA specific antibodies also facilitate phagocytosis of IAV particles by Fc receptor expressing cells and lysis of infected cells by NK cells, a process known as Ab-dependent cell-mediated cytotoxicity

(ADCC) (Hashimoto et al., 1983). Although effective, HA specific antibodies generally do not provide cross protection between different strains of IAV, those that do are Abs specific to the HA stalk rather than head (Ekiert et al., 2009; Ekiert et al., 2011; Sui et al., 2009). Stalk reactive HA Abs do not neutralize IAV by inhibiting the interaction between HA and sialic acid residues, instead stalk-reactive Abs may protect through several mechanisms including retaining newly formed HAs on the cell surface and thereby inhibiting virus budding (Tan et al., 2014). Additionally, Ab binding to HA stalks prevents the pH-triggered conformational change of IAV when the virus is taken up into the endosome by locking the HA trimer in a pre-fusion conformation, effectively trapping IAV within the endosome (Brandenburg et al., 2013; Ekiert et al., 2009; Tan et al., 2012). Moreover, HA stalk Abs sterically obstruct access of proteases to the basic cleavage site between the HA1 and HA2 subunits of HA, which is located in the stalk domain, and thereby block necessary HA conformational changes for fusion (Brandenburg et al., 2013; Ekiert et al., 2009) (Figure 1a-c). Finally, ADCC has been shown to potentiate the protective efficacy of stalk-reactive antibodies in vivo (DiLillo et al., 2014).

IAV infection also induces antibodies specific to other viral proteins. NA specific antibodies limit virus spread by binding to NA and blocking its enzymatic ability to cleave IAV virions from the cell surface. Furthermore, NA specific antibodies also contribute to clearance of virus-infected cells by ADCC (Mozdzanowska et al., 1999). Immunity against IAV M2 was first demonstrated in mice through the use of a therapeutic monoclonal Ab (mAb) raised against M2 (Treanor et al., 1990; Zebedee and Lamb, 1988). Finally, NP-specific antibodies can also contribute to protection against influenza virus infection, however, the exact

mechanism of protection remains to be elucidated. One study has suggested that NP specific antibodies can induce complement-mediated cell lysis of infected cells (Carragher et al., 2008; LaMere et al., 2011).

IAV infection also induces the proliferation and recruitment of CD8⁺, CD4⁺ and regulatory T cells (T regs) to the lung. CD4⁺ T cells or T helper cells differentiate into several different subsets, which are distinguished by the cytokines they secrete. Th1 cells produce IL-2 and type II IFN (IFN γ) and Th17 cells secrete IL-6 during IAV infection to stimulate the inflammatory response to IAV and block the function of regulatory cells. Th2 cells predominantly promote B cell responses through the production of IL-4 and IL-13 (Campbell and Koch, 2011; Lamb et al., 1982; McKinlay, 2001; Roman et al., 2002). T regs control both the T helper cell and the CD8⁺ T cell response through the secretion of IL-10 to protect the host from immune mediated damage (Sun et al., 2009).

Virus-specific CD8⁺ T cells recognize and eliminate IAV infected cells by interaction of their T cell receptor with influenza-peptide-MHC class I complexes on infected cells, and subsequent release of perforin and granzymes or the expression of apoptosis inducing ligands including Fas ligand (FasL) and tumour necrosis factor related apoptosis inducing ligand (TRAIL) (Brincks et al., 2011; Brincks et al., 2008a; Topham et al., 1997). Studies in humans have revealed IAV specific CD8⁺ T cells are primarily reactive to NP, M1 and PA proteins (Gotch et al., 1987; Jameson et al., 1998; McMichael et al., 1983; Townsend et al., 1985; Wang et al., 2007; Yewdell et al., 1985). These proteins are highly conserved, and therefore, the cellular arm of the adaptive immune response displays a higher degree of cross-reactivity between different IAV subtypes. However, viral epitopes that are recognised by cytotoxic T cells are under selective pressure, indeed many amino

acid substitutions observed during the evolution of H3N2 IAV strain were associated with escape from recognition by virus-specific CD8⁺ T cells (Berkhoff et al., 2007; Boon et al., 2004; Rimmelzwaan et al., 2004; Voeten et al., 2000).

The adaptive immune response to IAV is well studied and is vital to infection resolution. However, comparably less is known about the contribution of the innate immune system to controlling IAV infection. This is significant as the innate response initially controls viral replication and directs the quality and magnitude of the adaptive immune response through release of cytokines and the action of APCs.

1.4 Innate Immunity and IAV

The innate immune response to IAV is activated within minutes to hours of infection. Upon recognition, IAV induces chemokine and cytokine production from infected epithelial cells and tissue resident immune cells, such as alveolar macrophages (AMs). Cytokines can have autocrine, paracrine, and/or endocrine activity and, through receptor binding, can elicit a variety of responses, depending upon the cytokine and the target cell type. Cytokines can be subdivided into interferons (IFNs), which mediate antiviral immunity and activation of cells; interleukins (IL) which are involved in growth and differentiation of cell types and modulation of inflammation and fever; chemokines which mediate chemotaxis; colony-stimulating factors (CSF) which stimulate hematopoietic progenitor cell proliferation and differentiation, and the tumour necrosis factor (TNF) family which has potent proinflammatory capacity. In vivo, the initial release of inflammatory mediators is likely from infected lung epithelium and AMs. This

release results in the extravasation of pDCs, monocytes, NK cells and neutrophils from the peripheral blood across the endo-epithelial barrier into infected lung tissue (Ada and Jones, 1986). These cell types in turn produce additional cytokines, chemokines and other antiviral proteins. This arm of the immune response is vital to control IAV spread and to activate and direct the adaptive immune response. Due to the broad nature of the innate immune response, many features such as cytokines driving inflammation are redundant, however mice which have serious defects in their innate response, such as mice deficient for both IFN $\alpha\beta$ and IFN λ receptors or Signal Transducer and Activator of Transcription 1 (STAT1) deficient mice cannot control IAV replication and succumb to disease rapidly (Garcia-Sastre et al., 1998a; Mordstein et al., 2008).

1.4.1 Host recognition of Influenza A Virus

Influenza viral nucleotide components are recognised as pathogen associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), resulting in the initiation of the cellular immune response (Janeway, 1999). IAV PAMPs triggers the activation of at least four distinct PRRs: two members of the toll-like receptor (TLR) family (TLR3 and 7); one member of the RIG-I-like receptor (RLR) family known as retinoic acid-inducible gene I (RIG-I); and NOD-LRR- and pyrin domain containing 3 (NLRP3). RIG-I and NLRP3 are situated in the cytosol and sense IAV infection here, while the TLRs sense IAV products in the endosome. NLRP3 forms a multiprotein complex with an adaptor protein known as ASC (Apoptosis-associated speck-like protein containing a CARD) and pro-caspase 1. This complex is known as an inflammasome and in its active form it induces the secretion of the proinflammatory cytokine, IL-1 β and IL-

18 and pyroptosis of infected cells (Franchi et al., 2009). The activation of the NLRP3 inflammasome and production of IL-1 β usually requires two signals. The first signal is provided by PRR agonists and this priming signal results in transcriptional activation of the genes encoding pro-IL-1 β , pro-IL-18 and NLRP3. The second signal is triggered by host damage, which induces the activation and cleavage of caspase 1 and the secretion of mature IL-1 β and IL-18. Inflammasome activation and IL-1 β secretion leads chemokine production which enhance the recruitment of inflammatory cells including neutrophils and monocytes and or their maintenance in the lung following IAV infection (Pang and Iwasaki, 2011). It does not appear that NLRP3 interacts directly with IAV PAMPs, instead the NLRP3 is activated by common intracellular changes caused by IAV infection, at least three mechanisms have been described for IAV mediated activation of the NLRP3 inflammasome: PRR recognition of IAV ssRNA and induction of lysosomal maturation and reactive oxygen species (Allen et al., 2009), disturbances in intracellular ionic concentrations due to hydrogen ion flux through IAV's M2 ion channel (Ichinohe et al., 2010) and the presence of high-molecular-weight aggregates of IAV's virulence protein: PB1-F2 in lysosomes (McAuley et al., 2013). Of the PRRs involved in IAV detection, only NLRP3 does not appear to play a role in establishing an antiviral state, indeed studies in NLRP3 deficient mice infected with IAV did not reveal a difference in early virus load compared to wild type controls, instead NLRP3 appears to promote leukocyte recruitment and tissue repair (Allen et al., 2009; Thomas et al., 2009).

Characterised agonists for TLR3 are double stranded RNA (dsRNA) molecules, however due to the cellular RNA helicase UAP56, IAV replication does not generate dsRNA, instead it is hypothesised that TLR3 detects unknown RNA

structures found in IAV infected apoptotic cells which have been phagocytised (Schulz et al., 2005). TLR3 deficient mice have been shown to express decreased chemokine levels and leukocyte recruitment to the lungs during high dose IAV infection compared to wild type controls, however TLR3 deficiency does not impede secretion of IFN $\alpha\beta$ by pDCs or the adaptive immune response to IAV. TLR3 is therefore expendable for the control of primary IAV infection and the initiation of adaptive immune response during IAV infection (Diebold et al., 2004; Heer et al., 2007). Imai et al. demonstrated that heat inactivated H5N1 stimulated acid-induced acute lung injury in a TLR4 dependent manner (Imai et al., 2008). While a recent study has reported that human TLR10 expression is upregulated in primary macrophages upon influenza H5N1 viral infection and this upregulation was observed to substantially enhance IAV-RNP-induced activation of IL-8 expression (Lee et al., 2014). However it remains to be seen how important these TLRs are in maintenance of the immune response to IAV.

In contrast to PRR already discussed, TLR7 and RIG-I and their downstream signalling molecules are necessary for the induction of the antiviral response to IAV. Experiments performed in vivo have revealed that TLR7 and RIG-I signalling pathways are able to compensate for one another and it is only when both pathways were blocked through the genetic ablation of their downstream adaptor proteins: Myeloid differentiation primary response gene 88 (MyD88) and Mitochondrial antiviral-signalling protein (MAVS) (TLR7 and Rig-I respectively) that control of IAV replication is lost (Koyama et al., 2007). RIG-I distinguishes viral RNA from host RNA by recognition of triphosphate or diphosphate moieties on the 5'-end of viral RNA. IAV replication generates 5' triphosphate bearing viral ssRNA which is detected by RIG-I in the cell cytosol (Baum et al., 2010; Goubau et

al., 2014; Hornung et al., 2006; Pichlmair et al., 2006; Rehwinkel et al., 2010). Upon ligand binding, the helicase domain of RIG-I binds to adenosine triphosphate (ATP), which enables conformational changes that allows the Caspase Associated Recruitment Domains (CARDs) of RIG-I to be ubiquitinated by E3 ligases such as Tripartite Motif Containing 25 (TRIM25) (Gack et al., 2007) and RIPLET (Oshiumi et al., 2010). CARD ubiquitin promotes MAVS and RIG-I interaction and subsequent activation of interferon regulatory factor (IRF) -3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), leading to production of IFN $\alpha\beta$ and IFN λ and other cytokines (Jiang et al., 2011; Kowalinski et al., 2011; Luo et al., 2011). Until recently, it was unclear how this cytosolic receptor recognised viral RNA from a virus that replicates only in the nucleus, however one study has demonstrated that RIG-I localizes to antiviral stress granules which contain IAV RNA (Onomoto et al., 2012). In addition to RIG-I, another member of the RLR family: Melanoma Differentiation-Associated protein 5 (MDA-5) may also contribute to IAV PAMP induction of IFNs as small reductions in the IFN $\alpha\beta$ response has been observed in infected MDA-5-deficient cells compared to control cells (Husser et al., 2011; Kato et al., 2006; Loo et al., 2008).

TLR7 binds to single-stranded guanine (G) uracil (U)-rich RNA, found commonly in viral genomes. Unlike RIG-I recognition, TLR7 sensing of IAV infection does not require replication of the virus, instead IAV ssRNA is detected by TLR7 when taken up into the endosome (Diebold et al., 2004; Lund et al., 2004). Binding of ssRNA to TLR7 results in the recruitment of MyD88 through the Toll/IL-1R homologous region (TIR) domain located in the TLR7 cytoplasmic tail (Kawai and Akira, 2011). Similar to RIG-I signalling, activation of TLR7/MyD88 in pDCs results in IRF7 and NF- κ B activation and in secretion of high levels of IFN $\alpha\beta$ and

IFN λ . Like TLR7, TLR8 is situated in the endosome and until recently it was thought to be nonfunctional in mice and redundant with TLR7 in humans. However in 2006 Gorden et al. demonstrated that HEK293 cells transfected with murine TLR8 would respond to combination stimulation with a synthetic TLR8 agonist and poly T oligodeoxynucleotides and Martinez et al further demonstrated that TLR8-MyD88-dependent pDC activation played a critical role in innate immune control of vaccinia virus infection (Gorden et al., 2006; Martinez et al., 2010). In both these studies, TLR8 signalling was dependent upon PAMPs rich in poly(A)/T sequences which the IAV genome is not, therefore whether or not TLR8 contribute to recognition of IAV and subsequent orchestration of the immune response appears unlikely.

Overall, it appears TLR7 and RIG-I are redundant in IAV recognition at the level of a whole organism, however these pathways are not redundant within specific cell types: TLR7 deficiency in pDCs entirely ablated induction of the antiviral cytokines IFN α and β in response to stimulation with IAV, yet TLR7 deficiency did not affect secretion from cDCs, AMs, epithelial cells or mouse embryonic fibroblasts (MEFs). Instead, IFN $\alpha\beta$ production in response to IAV by these cell types was entirely dependent on MAVS signalling. Whilst TLR7 and RIG-I activate different downstream signalling pathways, they both lead to the translocation of similar transcription factors such as NF- κ B and AP-1. Of particular interest to this thesis, engagement of the TLR7 and RIG-I signalling cascades induces the phosphorylation of IRF7 and IRF3, which translocate from the cytosol to the nucleus and induce IFN $\alpha\beta$ and IFN λ expression (Diebold et al., 2004; Kato et al., 2005; Kawai and Akira, 2011; Koyama et al., 2007; Loo and Gale, 2011).

1.4.2 IFN $\alpha\beta$ and IFN λ response and IAV

IFN $\alpha\beta$ are a family of secreted cytokines that comprised of 13 partially homologous IFN α genes in humans (11 in mice), one IFN β gene (also one in mice) and several other family members (IFN- ω , - ϵ , - δ and - κ) (Pestka et al., 2004). IFN $\alpha\beta$ is secreted by cells to inhibit replication and spread of a range of viruses both in vivo and in vitro (Muller et al., 1994; Schneider et al., 2014b). The first report of IFN $\alpha\beta$ was made in 1954 by two Japanese virologists, Yasu-ichi Nagano and Yasuhiko Kojima, who observed that intradermal inoculation of rabbits with a UV treated tissue suspension of vaccinia virus-infected rabbit skin or testis would, within hours, protect cells at the site of inoculation from viral challenge (Nagano et al., 1954). Nagano and Kojima postulated the existence of some "facteur inhibiteur" (inhibitory factor) entirely independent of the virus itself that was protecting cells from vaccinia infection. However, Nagano and Kojima also observed that intraperitoneal (i.p.) injection of mice with their ultra-centrifuged supernatant elicited the production of virus-neutralizing Abs in mice, the authors could not reconcile the resistance of the rabbit skin cells to infection and this induction of virus specific Abs. They later found that Ab induction was due to viral contamination, however this initial ambiguity and publication of the article in French meant that Nagano and Kojima's discovery was unappreciated at the time. 3 years later, Isaacs and Lindenmann showed that addition of heat-inactivated IAV to chick chorioallantoic membrane fragments could induce the formation of an 'interfering' reagent that inhibited growth of live IAV when transferred to fresh membranes (Isaacs and Lindenmann, 1957). In their 1957 publication Isaacs and Lindenmann first coined the term "interferon," and this interfering agent was later

characterised as type I IFN or IFN $\alpha\beta$, although it is not inconceivable that Isaacs and Lindenmann could have been describing IFN λ .

There are many type I IFN family members, however only IFN α subtypes and IFN β are broadly expressed and have been demonstrated to have a clear antiviral role during IAV infection. All IFN $\alpha\beta$ subtypes act through a common, ubiquitously expressed, heterodimeric receptor, IFN $\alpha\beta$ R. IFN $\alpha\beta$ R is composed of a low affinity subunit known as IFNAR1 and a high affinity subunit, designated IFNAR2. Each receptor subunit contains an extracellular ligand-binding domain and an intracellular kinase domain which is associated with a Janus activated kinase (JAK), with IFNAR1 being associated with a tyrosine kinase 2 (TYK2) and IFNAR2 with JAK1. IFN $\alpha\beta$ subtypes bind first to IFNAR2 and this recruits IFNAR1, receptor heterodimerization then occurs and elicits activation of the JAKs and therefore tyrosine phosphorylation of STAT2 and STAT1 (Murray, 2007; Platanias, 2005). JAK/STAT signalling induces the three molecules STAT1, STAT2 and IRF9 to form the trimeric transcription factor ISGF3 (Interferon-stimulated gene factor 3), and it is ISGF3 that triggers the transcription of a diverse set of genes known as IFN stimulated genes (ISGs) and thereby establishes an antiviral state in stimulated cells (Darnell et al., 1994; Ivashkiv and Donlin, 2014; Sadler and Williams, 2008; Schneider et al., 2014b).

ISGs counteract IAV spread by diverse mechanisms. For example, Protein kinase R (PKR) is required for antiviral stress granule formation and therefore RIG-I sensing of IAV. Radical S-Adenosyl Methionine Domain Containing 2 (RSAD2, also known as viperin) promotes TLR signalling by recruiting IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) to lipid bodies and by inducing nuclear translocation of IRF7 and 2'-5'oligoadenylate

(OAS) is activated by dsRNA to degrade ssRNA. The IFN-inducible transmembrane protein 3 (IFITM3) blocks the release of viral contents into the cytosol and as mentioned previously, murine Mx1 interacts with IAV NP and polymerase complex PB2, leading to a blockade of viral replication. Furthermore, IFN $\alpha\beta$ themselves also induce a positive feedback loop and trigger the expression of IFN-inducing proteins such as RIG-I, NF- κ B and STATs as well as certain IFN α subtypes (Desai et al., 2014; Haller et al., 1979; Sadler and Williams, 2008; Schneider et al., 2014b; Trinchieri, 2010).

Discovered in 2003, Type III IFNs (IFN λ 1, 2 and 3) are induced during viral infection via the same pathways as IFN $\alpha\beta$ and employ an almost identical signalling cascade to activate transcription of ISGs (Kotenko et al., 2003; Sheppard et al., 2003). However, IFN λ utilizes a separate receptor complex with a limited tissue distribution, compared to the ubiquitously expressed IFN $\alpha\beta$ R (Mordstein et al., 2008; Sheppard et al., 2003; Sommereyns et al., 2008). It is thought that IFN λ is more important than IFN $\alpha\beta$ for host protection during IAV infection as it is particularly secreted by epithelial cells, the main cell type used by IAV for viral replication. However, studies which infected both IFN $\alpha\beta$ R $^{-/-}$ and IFN λ R $^{-/-}$ mice with IAV found that only the absence of both receptors caused dramatic loss of virus control (Mordstein et al., 2008; Mordstein et al., 2010). Thus, IFN $\alpha\beta$ and IFN λ are able to redundantly induce antiviral signalling pathways to protect against IAV induced disease.

Arguably, the best indication of the potent anti-influenza effects of IFN $\alpha\beta$ and IFN λ is the fact that part of the viral non-structural (NS1) protein is dedicated to counteract their induction and signalling. NS1 is the common factor by which all IAVs antagonize host immune responses. It is a multifunctional protein which has

been demonstrated to perform a wide range of functions (Egorov et al., 1998; Garcia-Sastre et al., 1998b; Kochs et al., 2007). NS1 facilitates efficient virus replication by contributing to the control of viral RNA synthesis, splicing and translation, regulation of virus particle morphogenesis and suppression of host apoptotic and immune response, in particular the IFN-mediated antiviral response to IAV infection (Hale et al., 2008). The mechanisms of NS1 antagonism of IFN $\alpha\beta$ and IFN λ varies between IAV strains; NS1 from the common lab adapted IAV strain A/Puerto Rico/8/34 (PR8) prevents virus-mediated activation of the IRF-3, NF- κ B and other transcription factors, which are important for induction of IFN $\alpha\beta$ and IFN λ (Ludwig et al., 2002; Talon et al., 2000; Wang et al., 2000). NS1 also limits activation of RIG-I by sequestering dsRNA (Donelan et al., 2003; Pichlmair et al., 2006; Steidle et al., 2010) and by specific inhibition of RIG-I ubiquitination by TRIM25 (Gack et al., 2007). Engineered IAV strains which do not express NS1 (delNS1 IAV) induce large amounts of IFN in infected cells and are consequently attenuated in IFN-competent systems. Indeed, both Diebold et al. and Kallfass et al. found that NS1 restricted IFN producing capabilities of AMs and cDCs (Diebold et al., 2003; Kallfass et al., 2013). Mordstein et al. demonstrated that while delNS1 IAV strain hvPR8-delNS1 was entirely non-pathogenic in wild type (Wt) mice it did cause disease in mice genetically deficient for both IFN $\alpha\beta$ R and IFN λ R (Mordstein et al., 2008). Due to the comparatively more recent discovery of IFN λ , NS1 action has been characterised in the context of IFN $\alpha\beta$, however it is important to state that the mechanisms employed by NS1 to antagonise IFN $\alpha\beta$ induction also antagonise IFN λ production and secretion. Indeed, delNS1 IAV strains only display high pathogenicity in mice lacking antiviral mediators involved in both IFN $\alpha\beta$ and IFN λ induction or downstream signalling, such as PKR (Bergmann et al., 2000;

Kochs et al., 2007) or STAT1 (Garcia-Sastre et al., 1998a). Furthermore, Mordstein et al. found that hvPR8-delNS1 remained non-pathogenic in IFN $\alpha\beta$ R^{-/-} mice, demonstrating commonality between IFN $\alpha\beta$ and IFN λ not only in antagonism by NS1 but also in their redundancy for host protection in IAV infection (Mordstein et al., 2008).

The antiviral action of IFN $\alpha\beta$ is well characterised; however IFN $\alpha\beta$ signalling goes far beyond induction of an antiviral state, as these cytokines elicit and direct many features of both the innate and adaptive immune response, thereby enhancing the immune response for more effective resolution of infection. IFN $\alpha\beta$ acts in both an autocrine and paracrine manner to signal almost every cell type in the body. By contrast, the effects of IFN λ are largely limited to non-haematopoietic cells, owing to the restricted expression of IFN λ R (Mordstein et al., 2008; Sheppard et al., 2003; Sommereyns et al., 2008).

IFN $\alpha\beta$ modulates the development of adaptive immunity by inducing cDC differentiation and maturation as well as enhancing cell-surface expression of MHC molecules and co-stimulatory molecules such as CD80 and CD86 and promoting cDC cross-presentation of antigens during viral infections (Lapenta et al., 2003; Montoya et al., 2002; Santini et al., 2000; Santodonato et al., 2003). IFN $\alpha\beta$ enhances CD4⁺ T cell clonal expansion and survival in Lymphocytic choriomeningitis virus (LCMV) infection (Havenar-Daughton et al., 2006) and in a model for induction for Abs against soluble antigen, IFN $\alpha\beta$ R signalling was important on both CD4⁺ T cells and on B cells for optimal Ab responses (Le Bon et al., 2006). Moreover, in IAV infection, early activation of respiratory tract B cells has been shown to be mediated by IFN $\alpha\beta$ R, and this activation dictated the quality and quantity of the antibody response (Chang et al., 2007b; Coro et al., 2006).

IFN $\alpha\beta$ has also been reported to play a role in Ab class switching between IgG subtypes during IAV infection (Heer et al., 2007). With regard to CD8⁺ T cell function, cytotoxicity is positively regulated by IFN $\alpha\beta$ (Curtsinger et al., 2005), and IFN $\alpha\beta$ is also upstream of recruitment of monocyte precursor cells that differentiate into DCs which promote proliferation of IAV-specific CD8⁺ T cells in the IAV infected lung (Aldridge et al., 2009). Similarly, IFN $\alpha\beta$ also directly induces activation and expression of cytolytic effector functions and type II IFN (IFN γ) production by NK cells in IAV infection (Hwang et al., 2012). Generally, IFN $\alpha\beta$ has been shown to enhance immune cell recruitment and activation both directly, and through the induction of other proinflammatory cytokines such as IL-6, IL-12, monocyte chemotactic protein (MCP)-1 (also known as CC chemokine ligand (CCL) 2), Interferon gamma-induced protein 10 (IP-10, also known as C-X-C motif chemokine 10 (CXCL10)) and IL-15 (Parlato et al., 2001; Seo et al., 2011; Trinchieri, 2010; Yamaji et al., 2006). However, IFN $\alpha\beta$ can also acts as an immunosuppressant. IFN $\alpha\beta$ signalling induces IL-1R antagonist (IL-1Ra), which binds non-productively to IL-1R and in this way inhibits proinflammatory action of IL-1 β (Novikov et al., 2011; Tilg et al., 1993). IFN $\alpha\beta$ mediated upregulation of Programmed death-ligand 1 (PD-L1) and IL-10 in chronic LCMV infection impaired CD4⁺ T cell action and thereby supported viral persistence (Teijaro et al., 2013; Wilson et al., 2013). IFN $\alpha\beta$ are therefore pluripotent cytokines with wide reaching effects on the immune system.

As all IFN $\alpha\beta$ subtypes exclusively signal through the IFN $\alpha\beta$ R, many studies designed to elucidate the role of type I IFNs in influenza infection have been conducted under conditions of IFN $\alpha\beta$ R deficiency. In vitro studies using IFN $\alpha\beta$ deficient cell lines have shown an increase in susceptibility to IAV infection and

replication (Garcia-Sastre and Biron, 2006; Garcia-Sastre et al., 1998a; Isaacs and Lindenmann, 1957; Koerner et al., 2007; Matzinger et al., 2013). However, investigation of IFN $\alpha\beta$ R deficiency in restricting IAV infection in vivo is less clear, and some studies report little to no increase in IAV induced morbidity and mortality of IFN $\alpha\beta$ R^{-/-} mice (Mordstein et al., 2008; Price et al., 2000). Increased susceptibility to influenza-induced disease in IFN $\alpha\beta$ R^{-/-} mice has been recorded, yet this has been in the context of high viral doses in mice expressing functional Mx1 protein (Koerner et al., 2007) or in studies where influenza viruses used were capable of causing a systemic infection (Garcia-Sastre et al., 1998a; Szretter et al., 2009). Differences in influenza strain or dose and use of either IFN $\alpha\beta$ R^{-/-} or STAT1^{-/-} mice as models of IFN signalling deficiency on C57BL/6, 129, CD1 or mixed mouse backgrounds mean that comparison between (and even within) these studies is difficult. In particular, the use of STAT1^{-/-} to specifically ablate type I IFN signalling is problematic. While it was reasonable in the early studies to assume that in STAT1^{-/-} mice IFN $\alpha\beta$ R signalling was specifically ablated, it is now known that STAT1 acts downstream of not only IFN $\alpha\beta$ R but also downstream of the receptors for IFN γ and IFN λ as well as a number of other cytokines that have wide-ranging effects, such as IL-6, IL-10 and IL-27 (Casanova et al., 2012). Thus, the 'IFN $\alpha\beta$ ' mediated protection claimed to be lost in these studies which have used STAT1^{-/-} mice as a model for IFN $\alpha\beta$ signalling is likely a result of a combined effect on a range of cytokines.

Collectively, IFN $\alpha\beta$ and IFN λ are vital for early control of IAV replication, and IFN $\alpha\beta$ is an important stimulator of both the innate and adaptive immune response. In theory, all cell types can make IFN upon recognition of, or infection with appropriate pathogens. Primarily AMs but also AECs, pDCs, cDCs and

monocytes produce IFN $\alpha\beta$ and IFN λ during IAV infection (Cheung et al., 2002; Crotta et al., 2013; Hogner et al., 2013; Ioannidis et al., 2013; Jewell et al., 2007; Kallfass et al., 2013; Kaminski et al., 2012). Production of IFN $\alpha\beta$ and IFN λ in response to IAV is not dependent upon productive infection in immune cells. Indeed productive infection results in the generation of the viral protein NS1 that can block signals required for IFN induction (Diebold et al., 2003; Kallfass et al., 2013). Given their important role in host immunity to IAV it is unsurprising not only that IFN $\alpha\beta$ and IFN λ can be considered redundant with one another but also their induction is achieved through a variety of PRRs (as discussed) and that they are made by many cell types.

Human mortality to IAV infections, particularly highly pathogenic IAV strains, is associated with production of proinflammatory cytokines and tissue damage (de Jong et al., 2006; Louie et al., 2009; Peiris et al., 2004). As type I IFN is a potent immunomodulator, many studies have assessed the contribution of IFN $\alpha\beta$ to inflammation during IAV infection and reported that IFN $\alpha\beta$ levels in some cases correlates directly and in others inversely with host pathology. There are only a few studies on experimentally or clinically IAV infected humans that have assessed the levels of IFN $\alpha\beta$. Hayden et al. assessed the proinflammatory cytokine response to experimental infection of humans with IAV in nasal washings and found that IFN α levels positively correlated to symptom severity (Hayden et al., 1998). While studies that assessed IFN $\alpha\beta$ concentrations in the serum often did not detect these cytokines, one study did detect IFN α in serum samples of patients clinically infected with SO-IV and found that levels were lower in patients with severe disease compared to patients with milder disease presentation (Agrati et al., 2010). The difficulty of detecting IFN $\alpha\beta$ in serum samples of even in the critically

ill indicates that presence of this potent immunomodulating cytokine is tightly controlled.

Cheung et al found IFN β to be one of the first cytokines secreted by H5N1 infected human macrophages, and this preceded induction of other proinflammatory cytokines and chemokines, such as MCP-1, Macrophage Inflammatory Protein-1 β (Mip-1 β also known as CCL4), and IL-12. Conversely, in the same study, lower pathogenicity influenza strains incited a lower IFN β response, which correlated with diminished transcription of proinflammatory cytokines, however a causal relationship by blocking IFN $\alpha\beta$ was not established (Cheung et al., 2002). Furthermore, avian IAV strains have also been shown to induce dramatic and sustained expression of IFN $\alpha\beta$ in infected lung tissue of nonhuman primates and associated with severe necrotizing bronchiolitis and alveolitis (Baskin et al., 2009). In contrast, a study on the highly pathogenic 1918 influenza strain found that higher susceptibility in infected cynomolgus macaques correlated with low IFN $\alpha\beta$ induction when compared with a lower pathogenicity strain of IAV (Kobasa et al., 2007). H5N1 was also demonstrated to attenuate the IFN $\alpha\beta$ response in a polarized human bronchial epithelial cell model (Zeng et al., 2007).

Although informative, the above-mentioned studies are correlative. The role of type I IFNs during IAV infection, therefore, is highly controversial. It remains to be elucidated whether this cytokine drives not only an antiviral response, but also promotes pathogenicity during influenza infection. Furthermore, it is unknown whether IFN $\alpha\beta$ contributes to inter-individual differences in influenza susceptibility.

1.4.3 Proinflammatory cytokine response to IAV

Aside from IFN $\alpha\beta$, many other cytokines are secreted in response to IAV infection. As mentioned, these proteins are secreted by all pulmonary cell types to direct the host immune response to IAV. IFN γ is secreted by NK and T cells and assists the immune response to IAV by promoting NK cell killing of IAV infected cells, Ab secretion from B cells, activation of and antigen presentation by macrophages, activation of inducible Nitric Oxide Synthase (iNOS) and induction its own suite of ISGs (Graham et al., 1993; Schroder et al., 2004). IL-1 family members such as IL-1 β enhance IgM antibody responses and recruitment of CD4⁺ T cells to the site of infection, tissue repair and contribute to the inflammasome response (Schmitz et al., 2005; Thomas et al., 2009). IL-6 and its receptor (IL-6R) form a complex to achieve trans-signalling. This signalling is pro-inflammatory and modulates host fever, secretion of chemokines and T-cell proliferation (Scheller et al., 2011). TNF α has been shown to upregulate expression of molecules involved in recognition of IAV including RIG-I in in vitro experiments (Matikainen et al., 2006), and TNF receptor signalling has been shown to regulate the magnitude of the CD8⁺ T cell response during IAV infection (DeBerge et al., 2014). In contrast to proinflammatory cytokines, IL-10 is the canonical anti-inflammatory cytokine that acts on all cell types to dampen chemokine secretion and cellular activation (Couper et al., 2008), and IL-9 has been implicated in repair of the epithelial cell layer late in the infection (Monticelli et al., 2011).

Chemokines are released by a variety of cells in response to IAV infection in order to mediate the recruitment of immune cells to the lung. While cytokines generally exert pleiotropic effects, attraction of immune cells by many chemokines is selective for specific cell types. For example, MCP-1, CXCL8 (also known as IL-8),

and Eotaxin are major chemoattractant factors for monocytes, neutrophils and eosinophils, respectively (Dawson et al., 2000; Hammond et al., 1995; Rothenberg et al., 1996). In contrast, IP-10 binds to CXCR3 on cells and induces chemotaxis of a variety of cell types, particularly T cells and neutrophils (Dufour et al., 2002).

Through the recruitment, activation and maintenance of both innate and adaptive immune cell types, cytokines and chemokines control and clear IAV infection from the lung. However studies into human IAV infection, particularly into high severity cases of H5N1 infection, have demonstrated a clear direct correlation between disease severity and the magnitude of inflammatory response. Termed the cytokine storm, severe IAV infection in humans is characterised by aberrant cytokine and chemokine responses that associate with monocytic infiltration and destruction of the epithelial layer (Arankalle et al., 2010; de Jong et al., 2006; Hayden et al., 1998; Kaiser et al., 2001; Louie et al., 2009; Peiris et al., 2004). In particular, strong correlations between IL-6, IFN α and TNF α levels and the severity of disease symptoms in humans have been observed, not only in clinical cases but also in experimental infection of humans with seasonal IAV. Interestingly, disease symptoms of patients in these studies did not always directly correlate to viral load (Agrati et al., 2010; Arankalle et al., 2010; Hayden et al., 1998; Kaiser et al., 2001; Peiris et al., 2004). High serum levels of IL-6, TNF α and IFN γ were recorded in patients during the H5N1 outbreaks in Hong Kong (1997), and individuals infected with H5N1 in the early 2000s had elevated serum concentrations IP-10, MCP-1, IL-8 and monokine induced by IFN γ (MIG), compared to patients with seasonal IAV (Beigel et al., 2005; de Jong et al., 2006; Peiris et al., 2004). Similarly, a study on serum samples collected from H1N1 infected individuals revealed that IP-10 and Regulated on Activation, Normal T Expressed

and Secreted (RANTES or CCL5) were elevated, while another study found raised levels of IL-1RA, IL-2, IL-6, Mip-1 α (also known as CCL3), Mip-1 β , and IL-10 in the plasma of patients exhibiting severe H1N1 IAV induced disease (Arankalle et al., 2010; Lichtner et al., 2011). As previously mentioned, in vitro studies have also demonstrated superior induction of IFN β and TNF α by H5N1 viruses, compared to H3N2 and H1N1 AIV strains, in human macrophages (Cheung et al., 2002). In contrast to the aforementioned studies, Agrati et al demonstrated an inverse correlation between IFN α and MCP-1 concentrations in patient plasma and disease severity, correlating an impaired production of these cytokines in patients with a worse clinical outcome (Agrati et al., 2010).

Animal models have allowed for the investigation of IAV induced cytokine storm. Compared to infection with a low pathogenicity strain, 1918 IAV-infected macaques experienced an overly aggressive innate immune response characterised by strong upregulation of IL-6, IL-8, MCP-1, and RANTES gene expression in the lung and elevated levels of most of these cytokines in the sera (Kobasa et al., 2007). Similar results were also reported in murine infection with reverse mutant 1918 IAV: pulmonary concentrations of IFN γ , TNF α , MCP-1, Mip-1 α , Mip-1 β , Mip-2 (also known as CXCL2), Mip-3 α (aka CCL20), IL-1, IL-6, IL-12, IL-18, and granulocyte colony-stimulating factor (G-CSF) were found to be elevated compared to levels found in infection with low pathogenicity IAV (Kash et al., 2006; Kobasa et al., 2004; Tumpey et al., 2005a). H5N1 infection of macaques also led to prolonged expression of MCP-1 and IP-10 and other cytokine and chemokine genes, compared to infection with seasonal IAV (Baskin et al., 2009). Additionally, SOIV-H1N1 infected macaques exhibited an increase in IL-6, TNF, and IL-1 β gene

expression in the lungs and pronounced levels of IL-6 and MCP-1 in the plasma (Safronetz et al., 2011).

In experimental mouse models, genomic deletion of TNFR in mice infected with 1918 H1N1 demonstrated that signalling through this receptor was intimately linked to the massive inflammatory response, interestingly, increased survival of 1918 infected TNFR^{-/-} mice associated with a decrease in IFN-signalling-related antiviral gene expression (Belisle et al., 2010). Similarly, in another study, TNFR deficiency significantly reduced morbidity compared to wild type controls in IAV infection with two strains of H5N1 that exhibit high and low pathogenicity in mice, yet deficiency did not affect viral replication and spread, or ultimate disease outcome. This same study also infected mice deficient in IL-6 and found that deficiency for these cytokines individually did not alter disease outcome in either high or low pathogenicity settings. This data also confirmed earlier work performed by Kozak et al. (Kozak et al., 1997; Szretter et al., 2007). Finally, the latter study also found that IL-1R deficient mice exhibited heightened morbidity and mortality in low pathogenicity IAV infection, yet this difference was not observed with the more virulent IAV strain (Szretter et al., 2007). Schmitz et al. also demonstrated higher mortality of mice deficient in IL-1R, interestingly ablation of IL-1R signalling protected mice from IAV induced granulocytic inflammation, yet associated with lower IgM and CD4⁺ T cell response to IAV, leading to host mortality (Schmitz et al., 2005).

IL-10 deficiency during IAV infection augmented Th17 related proinflammatory cytokines and IL-17 producing CD4⁺ T cells. Therefore, IL-10 mediated dampening of Th17 responses increased host susceptibility to severe disease (McKinstry et al., 2009). In contrast to this, Sun et al. demonstrated that

mAb blockade of IL-10R during IAV infection lead to lethal pulmonary inflammation and consequent host mortality (Sun et al., 2009). IL-10 action may impede clearance of a pathogen however this can be considered a trade off for protection from host immune cell mediated tissue damage.

Interruption of C-C chemokine receptor type 5 (CCR5) signalling was also shown to increase IAV susceptibility in mice. Dawson et al. found that CCR5 deficient mice suffered increased accumulation of macrophages in the lung and higher pulmonary levels of the proinflammatory cytokines MCP-1, IP-10 and RANTES, and this associated with host mortality (Dawson et al., 2000). CCR5 mediated protection from severe IAV induced disease is likely through RANTES-CCR5 interaction, as RANTES deficient mice were also susceptible to IAV induced disease, while mice deficient for Mip-1 α , another known ligand of CCR5, exhibited either comparable disease progression to wild type controls or lower levels of IAV induced inflammation (Cook et al., 1995; Szretter et al., 2007; Tyner et al., 2005). Wang et al. demonstrated IP-10 to be upstream of lung damage in severe H1N1 infection and blockade of this cytokine by either genetic ablation or mAb neutralisation lowered IAV induced disease burden, significantly Wang et al. correlated their results with elevated concentrations of IP-10 in the sera of humans hospitalised due to SO-IV infection (Wang et al., 2013).

Clinical outcome of CCR2 deficiency in mice varies depending on experimental setting. In both IAV and RSV infection, AMs secrete IFN $\alpha\beta$, and this induces MCP-1 secretion mediating IMcs recruitment to the lung. IMcs have potent inflammatory and antiviral activity and in this way can promote the immune response to respiratory viruses (Aldridge et al., 2009; Dessing et al., 2007; Goritzka et al., 2015). However, overexpression of MCP-1 in the lung can lead to severe

disease in IAV infected mice, characterised by monocyte influx, lung tissue damage and ultimately, host mortality (Lin et al., 2008). How MCP-1 mediated IMC recruitment modulates IAV induced disease outcome is discussed in later sections.

There does not appear to be a pivotal cytokine storm mediator, collectively these studies in animal models demonstrate that there is yet to be a setting where complete absence of a specific cytokine or its cognate receptor entirely ablates IAV induced cytokine storm. It is interesting to note that IFN $\alpha\beta$ is known to induce proinflammatory cytokine secretion from many cell types and as discussed, IFN $\alpha\beta$ in some studies correlates with high levels of other IAV induced proinflammatory cytokines and severity IAV induced disease. However, these observations are only correlative and do not always hold true, some studies find that there is a negative correlation between IFN $\alpha\beta$ levels and IAV induced cytokine storm and consequent disease severity. Further study is required to ascertain whether or not there is a casual link between IFN $\alpha\beta$, proinflammatory cytokine secretion and disease severity in IAV infection.

1.4.4 AMs and IAV

AMs are long-lived, terminally differentiated tissue resident cells capable of limited cellular division. They occupy a unique environmental niche, positioned in direct contact with the external atmosphere and the lung epithelial cell layer. AMs are vital for the maintenance of surfactant homeostasis in the alveolar space as well as clearing particulate antigens and epithelial cells which have undergone apoptosis from the airways by phagocytosis (Trapnell and Whitsett, 2002). As they occupy a space within the tissue which is constantly and directly exposed to the external environment, AM inflammatory responses must be tightly regulated, and

as such resting AMs produce only low levels of inflammatory cytokines and are less phagocytic than their counterparts in other tissues (Holt et al., 2008). One such regulatory mechanism is the expression of prostaglandin E2 (PGE₂) (Coulombe et al., 2014). Yet, AMs are among the first cells in the respiratory tract to detect and respond to IAV and therefore play a pivotal role in mounting effective innate and adaptive responses (Kim et al., 2008; Tumpey et al., 2005b).

Upon activation by IAV, AMs convert into highly phagocytic cells, as aside from cytokine secretion AMs are also tasked with clearance of apoptotic host cells from the airways and this function is essential to limit host pathology (Hashimoto et al., 2007). Genetic deletion of the colony-stimulating factor Granulocyte-macrophage colony-stimulating factor (GM-CSF) results in mice that are deficient in AMs. Infection of GM-CSF deficient mice with IAV lead to obstruction of alveoli with aggregates of eosinophilic material. Moreover, the BAL fluid of these mice was highly enriched in dead cells and cellular debris, indicating impaired clearance of apoptotic cells (Schneider et al., 2014a). Schneider et al also found that genetic deletion of GM-CSF or AM depletion from Wt mice impaired gas exchange in the lung and resulted in fatal hypoxia associated with severe morbidity to IAV infection, yet viral clearance was only moderately affected (Schneider et al., 2014a).

RIG-I mediated recognition of IAV elicits secretion of a wide range of proinflammatory cytokines by AMs. Significantly, this proinflammatory cytokine secretion is enhanced in highly pathogenic strains of IAV. In two independent studies Cheung et al. and Zhou et al. both showed that infection of human monocyte derived macrophages with H5N1 resulted in higher expressions of TNF α , IFN β , RANTES, MIP-1 α , MIP-1 β , MCP-1 and IP-10, than what was induced by

infection with seasonal IAV strains (Cheung et al., 2002; Zhou et al., 2006). Macrophages derived from blood monocyte have a more inflammatory phenotype than tissue resident AMs (Holt et al., 2008), therefore these experiments may be more indicative of the response of IMcs recruited to the lung during IAV infection. Nevertheless, Perrone et al demonstrated that H5N1 virus consistently elicited significantly higher levels of pro-inflammatory cytokines in whole lungs and primary human macrophages compared to other IAV strains (Perrone et al., 2008). AMs secrete these cytokines in order to combat IAV infection. As discussed, IFN β induces the antiviral state in AECs, and chemokines such as MCP-1, RANTES and IP-10 drive the recruitment of other immune cell types to the lung. In particular, MCP-1 induces significant recruitment of IMcs via CCR2 (Dawson et al., 2000), which differentiate into monocyte-derived DCs and macrophages leading to the marked expansion of the macrophage pool (Geissmann et al., 2010).

AM action early in IAV infection has been shown to be vital for protection from severe IAV induced disease. Depletion of AMs in pigs prior to, and throughout IAV infection lead to elevated clinical score and weight loss compared to nontreated controls. AM depletion led to lower levels of TNF α secretion and a decreased percentage of CD8⁺ T cells expressing IFN γ , culminating in higher virus loads and host pathology (Kim et al., 2008). Similarly, Tumpey et al. also demonstrated that depletion of AMs prior to, but not during, IAV infection of mice resulted in uncontrolled viral replication, blunted pulmonary levels of proinflammatory cytokines including IFN α , IFN γ and TNF α and this associated with a significant increase in IAV-associated mortality (Tumpey et al., 2005b). Although AMs have also been shown to drive host immunity to IAV by antigen presentation to T cells (Coulombe et al., 2014; Kim et al., 2008; Wijburg et al.,

1997), Schneider et al. attributed induction and maintenance of the CD8⁺ T cell response to IAV to CD103⁺ DCs rather than AMs (Schneider et al., 2014a). These studies demonstrate the integral role AMs play in the early control of virus infection, prior to the induction of adaptive responses. However, AM mediated immunostimulation is tightly regulated, as PGE₂ is up regulated by this cell type during IAV infection. PGE₂ action on AMs inhibits IFNαβ production from this cell type. Furthermore PGE₂ expression decreased AM capability to present antigen to CD8⁺ T cells. Collectively, PGE₂ mediated inhibition of AM proinflammatory function increased virus loads in the lung and host susceptibility to lethal IAV dose (Coulombe et al., 2014).

A conclusion that may be drawn from the above studies is that AM mediated induction of proinflammatory cytokines is protective for the host in IAV infection. However, induction of proinflammatory factors from AMs can have deleterious consequences in IAV infection. Nitric oxide synthase 2 (NOS2) and TNFα have both been shown to be secreted by AMs and also to associate with immunopathology in IAV. TNFα specific neutralizing Abs ameliorated lung lesions and increase survival time of CD1 mice infected with a lethal dose of IAV, however neutralisation of TNFα did not affect virus titers in the lung (Peper and Van Campen, 1995). Antioxidant treatment (which inhibits NOS2) of IAV-infected mice results in improved lung function and accelerated disease resolution, while genetic ablation of NOS2 in mice lowered IAV titres in the lung compared to Wt controls, and this associated with increased virus-specific IgG2a Abs (Jayasekera et al., 2006; Snelgrove et al., 2006). Furthermore, AMs may be upstream of these proinflammatory mediators as AM secreted MCP-1 mediates recruitment of IMCs which have also been demonstrated to produce the NOS2 and TNFα in response to

IAV infection (Aldridge et al., 2009; Lin et al., 2008), discussed further in a later section.

Although respiratory epithelial cells are the primary site of IAV replication, many studies suggest that IAV also infects AMs; however whether or not this infection results in productive replication in these cells is controversial. Rodgers et al found that seasonal strains of IAV could infect human AMs obtained by fiberoptic bronchoscopy, and NP antigen expression in AMs was observed, however no virus release was detected (Rodgers and Mims, 1982). Similar results were also recorded by other groups (van Riel et al., 2011; Yu et al., 2011). Yu et al. and van Riel et al. also compared infectivity of seasonal IAV and highly pathogenic H5N1 IAV strains. Both observed that H5N1 replicated productively in AMs and, as previously observed by Cheung et al and Zhou et al., higher pathogenicity of IAV strain correlated with higher proinflammatory cytokine secretion (van Riel et al., 2011; Yu et al., 2011). Similarly, infection of murine AMs with seasonal IAV was abortive (ie: no new virions were produced). It is thought that highly pathogenic avian IAV strains are able to generate productive infection in AMs, due to their expression of polybasic HA cleavage sites which are cleavable by ubiquitously expressed proteases (Stieneke-Grober et al., 1992). It is likely that high pathogenicity in H5N1 strains, characterised by an elevated inflammatory response, is intimately linked to the ability of this IAV strain to productively infect AMs, thereby augmenting activation of this cell type.

1.4.5 pDCs and IAV

Although most cell types produce both IFN $\alpha\beta$ and IFN λ to varying degrees, pDCs are characterized as exquisite IFN producers. pDCs were first discovered in

humans and were originally characterised in humans under a number of different pseudonyms: plasmacytoid monocytes (Facchetti et al., 1990; Facchetti et al., 1988), pre-DC2 (Grouard et al., 1997), and natural IFN-producing cells (Chehimi et al., 1989; Feldman and Fitzgerald-Bocarsly, 1990; Ronnblom et al., 1983; Sandberg et al., 1991; Starr et al., 1993), until properly defined as pDCs in 1999 (Cella et al., 1999; Siegal et al., 1999). 2 years later the existence of murine pDCs was confirmed (Asselin-Paturel et al., 2001; Bjorck, 2001; Nakano et al., 2001). Interestingly, these early definitions specify the key properties of pDCs: potent IFN production, secretory plasmacytoid morphology, and the ability to differentiate into cDCs.

pDCs sense IAV infection through TLR7 and are generally immune to IAV infection due to their strong propensity for IFN production (Diebold et al., 2004; Kato et al., 2005; Liu, 2005). Why pDCs are such potent IFN producers is still not entirely understood. IRF7 is required for IFN secretion from pDCs (Kumagai et al., 2009), and pDCs express high levels of IRF7 in the steady state compared to other immune cells (Dai et al., 2004; Izaguirre et al., 2003; Kerkmann et al., 2003). Furthermore, pDCs are capable of retaining PAMPS in the early endocytic compartment, thereby propagating IFN induction (Honda et al., 2005). There are also several surface receptors on pDCs which can alter IFN $\alpha\beta$ secretion, including SCARB2, DOCK2, Ly49Q and Siglec H (Gotoh et al., 2010; Guo et al., 2015; Puttur et al., 2013; Tai et al., 2008). Propensity of pDCs to generate such massive amounts of IFNs is therefore controlled on many levels.

In response to IAV, both human and mouse pDCs secrete massive amounts of IFN $\alpha\beta$ and IFN λ (Diebold et al., 2004; Koyama et al., 2007; Liu, 2005; Thitithanyanont et al., 2007). Diebold et al. demonstrated that IFN $\alpha\beta$ secretion from pDCs in vitro does not depend on live IAV activity but does require

endosomal recognition of IAV ssRNA by TLR7 (Diebold et al., 2004). This recognition of IAV both live and UV inactivated, by TLR7 in pDCs appears to be independent of pDC autophagy (Lee et al., 2007). pDC derived IFN $\alpha\beta$ can assist in establishment of the expression of antiviral genes in cells vulnerable to IAV infection such as AECs. Aside from IFNs, pDCs also have also been shown to secrete proinflammatory cytokines such as TNF α and IP-10 that assist the immune response during infection. Whilst pDCs are not as effective at taking up soluble or cellular antigens as cDCs, immature pDCs have been demonstrated to endocytose cellular material from live influenza-exposed cells, subsequently mature, and cross-present viral antigens very efficiently to specific CD8 $^{+}$ T cells (Lui et al., 2009). Furthermore, Langlois et al. demonstrate that pDCs eliminate virus-specific cytotoxic lymphocytes in the lung following pathogenic influenza virus infection, suggesting a pDC-mediated role in restoring immunologic homeostasis post-infection (Langlois and Legge, 2010). pDCs therefore affect many facets of the host immune response to IAV.

In some viral infections, pDCs and their secreted IFN $\alpha\beta$ constitute a critical aspect of host immunity. pDC mediated control of virus load is critical for host protection during mouse hepatitis virus (MHV) infection, herpes simplex virus 2 (HSV2) infection and early life infection with pneumovirus for mice (PVM) (Cervantes-Barragan et al., 2012; Cervantes-Barragan et al., 2007; Davidson et al., 2011). However, pDCs have been demonstrated to be nonessential for control of IAV infection. GeurtsvanKessel et al found that mAb mediated depletion of pDCs during IAV infection did not affect immune response to IAV: virus was cleared by 8dpi and surprisingly, IFN α concentrations in the lung were unaffected by depletion (GeurtsvanKessel et al., 2008). In another study using the same method

of pDC depletion during IAV infection observed decreased pulmonary levels of IFN α , reduction in lung virus burden, enhancement of mononuclear phagocyte progenitor generation, recruitment of cDCs, AMs and exudate macrophages to the lung and increased production of TNF- α and IL-6 from these cell types compared to non depleted controls (Soloff et al., 2012). However, on balance this did not alter disease outcome. Infection of Ikaros^{L/L} mice, which have no pDCs, with IAV demonstrated that pDCs were not required for generation of neutralizing Abs or IAV-specific effector and memory CD8⁺ T cells (Wolf et al., 2009). In contrast, Kaminski et al. found a protective role for TLR7 and pDCs in the severe H7N7 IAV infection, genetic ablation of TLR7 signalling or depletion of pDCs decreased survival of mice infected with H7N7 infected (Kaminski et al., 2012).

In vitro infection of human pDCs isolated from PBMCs with high and low pathogenicity IAV strains elicited secretion of IFN α and other proinflammatory cytokines including TNF α and IP-10. Similar to what was observed in AMs and monocyte derived macrophages, the IFN- α response induced by the high pathogenicity (H5N1) strains tested was several-fold higher than that which was induced by low-pathogenicity IAV strains. However, this did not follow for secretion of TNF α and IP-10, production of TNF α by pDCs was only slightly elevated in high pathogenicity IAV strains compared to low and IP-10 secretion was equivalent between all IAV strains tested (Sandbulte et al., 2008).

1.4.6 Monocyte derived cell types and IAV

In order to establish or replenish DC and macrophage populations at the site of IAV infection, monocytes are recruited from the bone marrow to the lung. Monocytes are a heterogeneous population of circulating hematopoietic cells that

originate from a common myeloid progenitor. Monocytes are broadly categorised based on their expression of the marker lymphocyte antigen 6C (Ly6C) as resident (Ly6C^{lo}) and inflammatory (Ly6C^{hi}). At baseline, resident monocytes circulate in the blood and act as sentinel cells, however upon infection, Ly6C^{hi} IMcs are signalled to egress from the bone marrow. This process is entirely dependent upon engagement of CCR2, primarily (however not only) by MCP-1 (Kurihara et al., 1997; Kuziel et al., 1997; Si et al., 2010).

Recruited monocytes and differentiated macrophages can be infected with IAV, however *de novo* synthesis of viral proteins has been shown to be interrupted before completion of the first replication cycle, and these cells die off by apoptosis within 48hrs of infection (Fesq et al., 1994). IAV is recognised by monocytic cell types through replication dependent and independent mechanisms (Hofmann et al., 1997; Kaufmann et al., 2001). Upon recognition, monocyte derived cell types, especially IMcs secrete proinflammatory cytokines such as IFN $\alpha\beta$, TNF α , IL-6 and IL-1 β ; chemokines including MCP-1, MIP-1 α , RANTES and IP-10; as well as reactive nitrogen intermediates through iNOS to combat IAV spread and promote host immunity (Gong et al., 1991; Hofmann et al., 1997; Julkunen et al., 2001; Kaufmann et al., 2001; Seo et al., 2011; Sprenger et al., 1996).

Depending on the virulence of the IAV strain used and the initial viral inoculate, IMcs can be protective or detrimental to the host. In a model for blocking monocytic recruitment by genomic ablation of the CCR2 gene, it was observed that CCR2^{-/-} mice had a reduced T cell expansion in the draining lymph nodes and this associated with elevated IAV presence in the lung (Dawson et al., 2000). Furthermore, chemical blunting of IMc recruitment using the drug pioglitazone also revealed that IMcs were required for an efficient CD8⁺ T cell response

(Aldridge et al., 2009). However, IMcs have also been shown to contribute to lung damage during severe IAV infection. CCR2^{-/-} mice as well as those treated with pioglitazone or a small molecule inhibitor for CCR2 all exhibit less lung damage than their wild type counter parts (Aldridge et al., 2009; Dawson et al., 2000; Herold et al., 2008; Lin et al., 2008; Lin et al., 2011). Although IMc recruitment is entirely dependent on CCR2, MCP-1, the primary ligand of CCR2, is not essential, as mAb blockade of MCP-1 or MCP-1^{-/-} mice results in a comparatively milder phenotype (Dessing et al., 2007; Narasaraju et al., 2010).

1.5 Epithelial cell death and clinical features of IAV infection

Modern histopathological analysis of autopsy samples from human influenza cases from 1918 revealed significant damage to the lungs with acute, focal bronchitis and alveolitis associated with massive pulmonary oedema, haemorrhage and rapid destruction of the respiratory epithelium (Taubenberger and Morens, 2006). Similar features have also been observed in modern samples taken from S-O IV H1N1 and H5N1 infected individuals (Louie et al., 2009; Mauad et al., 2010; Peiris et al., 2004). Cell death is a way for the host to restrict virus replication and spread, and infected cells either upregulate death receptors such as DR5, or are stimulated by other cell types to induce apoptosis. Many features of the inflammatory response such as IFN γ secretion can enhance this effect. However, excessive and prolonged inflammation may be detrimental to the host and contribute to the greater morbidity and mortality associated with influenza-induced inflammatory injury (Akaike et al., 1996; Monsalvo et al., 2011; Narasaraju et al., 2011). Kash et al found that IL-6, IFN $\alpha\beta$ and TLR response genes were more

strongly activated in severe IAV induced disease than mild and this associated with severe pulmonary pathology (Kash et al., 2006).

Apoptosis is one of the processes of programmed cell death that occurs in multicellular organisms. It is a highly regulated and controlled process that involves biochemical changes of the cell (including membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay) resulting in cell death. Unlike necrosis, where cellular contents are indiscriminately released, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage. Death-inducing members of the TNF superfamily, such as TRAIL and Fas ligand (FasL) have been shown to induce apoptosis of cells during IAV infection (Elmore, 2007; Holoch and Griffith, 2009).

FasL is the specific ligand for a type-I trans-membrane protein known as Fas, which induces cell death, in particular FasL/Fas signalling was shown to be essential for elimination of activated peripheral lymphocytes to terminate inflammation (Strasser et al., 2009). However, this must be controlled as pDCs have been shown to eliminate CD8⁺ T cells during IAV infection and in this way exacerbate, rather than ameliorate disease (Langlois and Legge, 2010). Similarly, Fas-dependent apoptosis has been demonstrated as a mechanism employed by the host to eliminate IAV infected cells (Fujimoto et al., 1998), yet DNA microarray analysis performed by Kash et al. found that FasL/Fas signal related genes in the lung is associated with IAV induced mortality in mice (Kash et al., 2006). Moreover, administration of recombinant FasL protein or Fas agonists causes acute lung inflammation and AEC apoptosis, while inhibition of the Fas/FasL signal by

treatment with a recombinant decoy receptor for FasL increases the survival rate of mice after lethal IAV infection (Fujikura et al., 2013; Matute-Bello et al., 2005; Matute-Bello et al., 2001a; Matute-Bello et al., 2001b). Interestingly, IFN $\alpha\beta$ signalling was demonstrated to be critical for induction of FasL, yet not Fas protein expression in the lung in immune cells as well as T-1 α^+ cell types (likely AECs and endothelial cells) (Fujikura et al., 2013).

IMcs and other cell types can cause tissue damage through the expression of the apoptosis inducing ligand TRAIL (Ellis et al., 2015; Herold et al., 2008; Hogner et al., 2013). Also known as Apo2L, TRAIL is a member of the TNF superfamily (TNFSF10) of cytokines and was initially characterised in the context of cancer research where it was shown to induce apoptosis specifically in transformed cells but not in normal, untransformed tissues (Holoch and Griffith, 2009). TRAIL is a type II transmembrane protein in which the carboxyl terminus with the receptor-binding domain protrudes extracellularly. This protein forms trimers, and TRAIL activity is further regulated by a zinc ion bound to a cysteine residue (Cys 230) at the trimer's interface (Bodmer et al., 2000). The role TRAIL plays in infectious disease is beginning to be appreciated. TRAIL can be a secreted or membrane bound ligand, which interacts with any of five TRAIL receptors in humans or mice. However, it is only TRAIL-R1 and TRAIL-R2 in humans and only DR5 in mice that contain death domains and therefore induce cell death on TRAIL engagement, while the remaining TRAIL receptors are considered decoys (Benedict and Ware, 2012; Schaefer et al., 2007). Ex vivo assessment of human macrophages has shown that TRAIL expression and secretion by this cell type is enhanced in severe disease and IAV infection of PBMCs induces TRAIL upregulation. Intriguingly, IFN $\alpha\beta$ has also been shown to stimulate TRAIL

upregulation on monocyte-derived macrophages in (Brincks et al., 2008b; Hogner et al., 2013; Santini et al., 2000). Furthermore, IAV infection of a human lung epithelial cell line increases cell susceptibility to TRAIL mediated apoptosis (Brincks et al., 2008b). Interestingly, in vivo interruption of TRAIL signalling, either by genomic deletion or mAb blockade during IAV infection, can be beneficial or detrimental to the host where TRAIL blockade can protect the host from immune mediated tissue damage however, in severe IAV infection TRAIL^{-/-} mice are more susceptible to IAV induced disease due to accumulation of cytotoxic CD8⁺ T cells in the lung (Brincks et al., 2008a; Brincks et al., 2008b; Herold et al., 2008; Hogner et al., 2013).

DR5 in mice and TRAIL-R1 and TRAIL-R2 all have a death binding domain; ligand engagement of these receptors therefore transduces an apoptotic signal into the cell. TRAIL binding induces the recruitment of signalling molecules by homologous protein-protein interactions, forming the DISC (death-inducing signalling complex) (Walczak and Haas, 2008). Ligand binding also induces conformational changes in the receptor, which lead to the recruitment of the adaptor molecule FADD (Fas-associated death domain) via homologous death domain-death domain interactions. FADD, in turn, can recruit inactive caspase 8 through so-called death effector domain interactions. Upon caspase 8 activation at the DISC, caspase 8 has two possible substrates: pro-caspase-3 and Bid. Cleavage and subsequent autocatalytic activation of caspase-3 directly triggers a caspase cascade, ultimately leading to apoptosis, whereas cleavage of the pro-apoptotic Bcl-2 family member Bid links TRAIL signalling to the intrinsic or mitochondrial apoptosis pathway (Youle and Strasser, 2008). As mentioned above, TRAIL mediated cell death has been observed in IAV infected mice and has been linked to

both positive and negative disease outcome, depending on the severity of IAV induced disease.

1.6 IAV and Host genetics

IAV infection in humans manifests as a range of symptoms depending on the infected individual, from asymptomatic responses, nasal congestion, fever, myalgia and head ache to severe cases of acute respiratory distress syndrome (ARDS) and organ failure (Cate, 1987; de Jong et al., 2006; Mauad et al., 2010; Peiris et al., 2004; Taubenberger and Morens, 2006). There are three main determinants of this variability: the intrinsic virulence of the virus, acquired host factors (such as immunological memory and comorbidity) and intrinsic susceptibility of the host (Horby et al., 2013). Evolution of host-pathogen interactions results in variants in host genes that ultimately associate with good or bad prognosis following infection. With the advent of genome wide association studies (GWAS) this concept is becoming easier to investigate, and elements of the human genome are beginning to be correlated with the different severity levels of many diseases including IAV-induced disease.

A seminal study published in 2008 conducted by Albright et al. examined the genealogy of a population from Utah, USA, over a period of 100 years (1904-2004). By assessing medical and genealogy records, the authors observed that both close and distant relatives of individuals who died of influenza had a significantly increased risk of dying of influenza themselves, consistent with a combination of shared exposure and genetic effects (Albright et al., 2008). This study is particularly compelling given the huge cohort of 4855 deaths assessed,

and as IAV induced mortality of genetically related individuals frequently did not occur close in time, it indicates that host genetics could be more important than host environment. This study gives strong evidence that susceptibility to severe IAV induced disease is heritable. In contrast, while Gottfredsson et al.'s study on victims of the 1918 IAV outbreak in Iceland over a 6-week period did find evidence of familial aggregation of IAV induced mortality, the authors concluded host genetics did not contribute to this IAV induced mortality (Gottfredsson et al., 2008). However, the Icelandic study was comparatively limited, having a ten-fold lower number of subjects and restricted to a single time period where shared exposure would be a strong confounding factor. Therefore, Albright et al. provide evidence that IAV-induced disease severity has a heritable component while Gottfredsson et al.'s study is inconclusive.

More generally, this last decade has seen the spread of H5N1 which, although highly virulent in avian species, has a relatively low rate of infection in humans. It has been observed that among the clusters of human infections with H5N1, the majority of cases have occurred in genetically related family groups (Horby et al., 2010; Olsen et al., 2005; Pitzer et al., 2007). Arguably, H5N1 familial aggregation could be attributed to non-genetic variation in household risk of exposure to H5N1 rather than host-genetic factors (Pitzer et al., 2007), however it is striking that 50 out of the 54 H5N1 infection clusters reported by 2010 were comprised only of blood relatives (Horby et al., 2010). Another general observation is that during SO-IV H1N1 outbreak there was a high rate of hospitalisation of individuals with Down's syndrome (DS). DS sufferers have a total or partial triplication of chromosome 21 and therefore represent a genetically defined population of individuals. During the 2009 SO-IV pandemic DS sufferers

were more likely to be treated with antivirals and intubated, compared to non-trisomic hospitalised individuals (Perez-Padilla et al., 2010).

The most well characterised region of genetic variability in the human genome is that of the Human leucocyte antigen (HLA) locus. HLA hyper-variability is in fact the basic mechanism used by the host to ensure the recognition of PAMPs and antigen presentation to T cells (Blackwell et al., 2009). Cell-mediated lysis of IAV infected cells is dependent on HLA specificities (Biddison and Shaw, 1979; McMichael, 1978; McMichael et al., 1977; Shaw and Biddison, 1979), and the HLA haplotype influences the magnitude and specificity of the cytotoxic T lymphocyte (CTL) response to IAV infection (Belz et al., 2000; Boon et al., 2002; Hertz et al., 2013). Currently, no genetic studies have been conducted to identify polymorphisms in HLA loci associated with susceptibility to IAV infection. Given the inherent diversity of HLA loci, the complex interaction of HLA in determining responses to infection and the linkage of HLA to other genes involved in innate immunity, such studies will be challenging (Horby et al., 2012).

More and more studies are emerging which demonstrate that severe IAV induced disease can result from single-gene inborn errors in immunity. In a study of one otherwise healthy child, who suffered life-threatening IAV induced illness during primary infection, Ciancanelli et al identified compound heterozygous null mutations in the gene coding for the IFN transcription factor IRF7. This loss of IRF7 function blunted the patient's IFN $\alpha\beta$ and IFN λ response to IAV and rendered stem cell-derived pulmonary epithelial cells from the patient highly permissive to IAV replication (Ciancanelli et al., 2015). Similar to Ciancanelli et al.'s study, Dupuis et al investigated two unrelated infants who both died of viral disease and found that this was associated with mutations in their alleles coding for the STAT1

transcription factor. Cells taken from these individuals were unresponsive to both IFN $\alpha\beta$ and IFN γ ; IFN stimulation did not activate STAT1-containing transcription factors and therefore ISG expression (Dupuis et al., 2003). Additionally, Duncan et al. identified a homozygous mutation in the high-affinity chain of the IFN $\alpha\beta$ R (IFNAR2) that rendered cells unresponsive to IFN $\alpha\beta$. The identified individual was highly susceptible to viral replication and succumbed to complications brought about by vaccination with attenuated virus (Duncan et al., 2015). Finally, Minegishi et al. identified a homozygous Tyk2 mutation in a patient that was acutely susceptible to microorganism invasion and this was attributed to defective signalling in the IFN $\alpha\beta$, IL-6, IL-10, IL-12, and IL-23 pathways (Minegishi et al., 2006).

Esposito et al. identified two mutations in the human TLR3 gene: the missense mutation F303S and the single nucleotide polymorphism (SNP) rs5743313, which were linked to influenza-associated encephalopathy (IAE) and to IAV-associated pneumonia, respectively (Esposito et al., 2012). CD55 is a protein thought to protect against IAV induced tissue damage, as this gene codes for a protective decay-accelerating factor that prevents cell damage caused by complement molecules and is particularly expressed in the lung (Osuka et al., 2006). A SNP (rs2564978) on the promoter region of CD55 associated with severe disease in S-O IV H1N1 infected individuals of Chinese and Japanese origin (Zhou et al., 2012).

In terms of genomic variance of specific proinflammatory cytokines, polymorphisms of in the *IL1A* gene and the *IL1B* gene have been positively correlated to higher susceptibility H1N1 infection (Liu et al., 2013). A single SNP in the *TNF* gene was found to correlate with severe IAV induced disease in one study

(Antonopoulou et al., 2012), though not in another (Ferdinands et al., 2011). A study on a small cohort of critically ill H1N1 infected patients indicated an increased prevalence in a 32 base pair deletion in the *CCR5* gene, however this was only in individuals of white ethnicity (Keynan et al., 2010). Finally, a hypothesis free study conducted by Zuniga et al. compared cases of S-OIV H1N1 with asymptomatic household contacts and identified four SNPs which associated with disease, three of which were within genes: an immunoglobulin Fc receptor (FCGR2A); a complement binding protein (C1QBP); and a protein that mediates the entry of replication protein A into the nucleus (RPAIN) (Zuniga et al., 2012).

Interestingly, in the context of hepatitis C virus (HCV) clearance in response to pegylated IFN α and ribavirin treatment or in spontaneous clearance, several SNP in the human IFN λ locus has been identified. Particularly protective in terms of HCV is the SNP rs368234815, which disrupts the open reading frame of *IFNL4*, thereby ablating expression of the IFN λ 4 protein (Hamming et al., 2013; Prokunina-Olsson et al., 2013). Additionally, another SNP: rs117648444 results in a nonsynonymous change in the coding region of *IFNL4*, where a proline residue is replaced by serine, resulting in two versions of IFN λ 4: the fully active IFN λ 4-P70 and a much less active IFN λ 4-S70 (Terczyńska-Dyla, 2015). Thus, HCV patients can be clustered into 3 groups, those that do not have IFN λ 4, those that have the less active form (IFN λ 4-S70) and those that have the fully active IFN λ 4-P70, with the presence and activity of IFN λ 4 inversely correlating to IFN α based treatment effectiveness (Terczyńska-Dyla, 2015). Intriguingly, the frameshift mutation in human IFNL4 was introduced approximately 55,000 years ago and was rapidly positively selected for, indicating that this gene may negatively impact the immune

response (Key et al., 2014), yet whether or not IFN λ 4 affects IAV induced disease severity remains to be studied.

In terms of mouse models, it has long been known that susceptibility to IAV varies between inbred laboratory mouse strains. Srivastava et al. assessed seven different inbred laboratory mouse strains and found a spectrum of disease, where severity correlated with virus load, high pulmonary concentrations of cytokines and chemokines and tissue damage (Srivastava et al., 2009). Further analysis of a selected resistant strain: C57BL/6J and a susceptible strain: DBA/2J revealed innate immune response genes were up regulated in both strains, but to a greater extent in the susceptible strain. Furthermore, crossing the two strains increased resistance, albeit with slightly higher IAV induced morbidity than the parental C57BL/6J strain, suggesting that susceptibility to IAV induced disease in mice is a polygenic trait. Similar results were also observed in an earlier study performed by another group (Alberts et al., 2010; Boon et al., 2009)

In a large study of 21 inbred mouse strains infected with avian IAV, Boon et al. also demonstrated a range of host susceptibility that spanned several logs of H5N1 inoculate. Further comparison of select mouse strains: C57BL/6, BALB/C, SM, 129/SvIm, A/J and DBA/2J, revealed that the DBA/2J strain was most susceptible to IAV induced disease, and this correlated to higher virus loads and inflammatory response in the lung. Gene expression and pathway analysis of select strains again demonstrated that differential gene expression primarily consisted of up-regulation of proinflammatory pathways in susceptible mouse strains, indicating the immune response is quantitatively but not qualitatively different between strains. No distinctive set of genes controlling replication or disease was identified in resistant mice (Boon et al., 2011). Infection of C57BL/6J (resistant

strain) and BALB/cByJ (susceptible strain) with a low pathogenicity IAV strain: H3N2 A/Hong Kong/X31/68 (X31) revealed a quantitative trait locus (QTL) on chromosome 6 that associated with influenza-induced slow-wave sleep patterns (Toth and Williams, 1999). Finally, use of recombinant inbred (RI) and select congenic strains of mice identified three loci that correlated with IAV induced disease severity. These genomic regions were: G-CSF on chromosome 5; IP-10 on chromosome 9, and IL-6 and Keratinocyte-Derived Chemokine (KC or CXCL1) on chromosome 18 (Trammell et al., 2012).

The earliest identification of a specific gene that affects IAV susceptibility in mice was the *Mx1* gene (Lindenmann, 1962). As previously mentioned, this gene was isolated from an outbred mouse strain and encodes for an IFN inducible protein that potently impedes IAV replication by inhibiting IAV PB2-NP interaction (Verhelst et al., 2012). Inbred laboratory mouse strains have deletions or a nonsense point mutation that results in a non-functional *Mx1* protein, and reconstitution of *Mx1* function in inbred strains of mice through breeding confers a significant increase in resistance to IAV infection (Salomon et al., 2007; Tumpey et al., 2007). The human homologue of this gene, *MxA*, has been identified and several polymorphisms in this gene have been described, however they have yet to be associated with severity of IAV induced disease (Horby et al., 2012).

In contrast to *Mx*, one gene that has been demonstrated to be important in both mice and humans for control of IAV replication is *IFITM3*. In an elegant study Everitt et al. were able to demonstrate that mice deficient for this gene developed severe viral pneumonia, which could be ameliorated if *IFITM3* was reintroduced into the system. Furthermore, the authors were able to identify a SNP in the human *IFITM3* gene (rs12252-C allele) that truncates the protein, leading to reduced

restriction of virus replication in vitro. This SNP conferred increased permissiveness to replication of a range of IAV strains in human lymphoblastoid cell lines and significantly, was found in higher prevalence in individuals hospitalised for H1N1 infection, strongly demonstrating that IFITM3 plays an important role in resistance to IAV (Everitt et al., 2012; Weidner et al., 2010). More recently, Zhang et al. have extended Everitt et al.'s study in Northern Europeans by assessing the frequency of the IFITM3 rs12252-C allele SNP in a Han Chinese population hospitalised for SO-IV infection. The authors found the susceptibility conferring rs12252-C allele in 69% of hospitalised patients with severe SO-IV induced disease, compared to only 25% in patients who were identified as having only mild infection (Zhang et al., 2013).

1.7 Study Rationale

Severe IAV induced disease is characterised by cytokine storm and tissue damage, and although this is intimately linked to IAV infection, it is intriguing to note that viral load in the lung sometimes correlates with symptom severity and in other settings it does not (Agrati et al., 2010; Arankalle et al., 2010; Hayden et al., 1998; Kaiser et al., 2001; Peiris et al., 2004). Understanding what drives the cytokine storm and therefore damage to the respiratory epithelium is vital for future development of treatment for IAV induced disease. One of the challenging clinical questions about IAV induced disease is why disease severity can vary so much between individuals. As discussed, there is evidence that variations in host genetics play a significant role in determining the susceptibility of an individual to IAV infection and subsequent disease outcome.

IFN $\alpha\beta$ are the prototypical antiviral cytokines and thought to be purely protective in IAV infection. However, given their potent immunomodulator capabilities, this family of cytokines could also drive the cytokine storm observed in severe IAV infection. As inter-individual differences in IFN $\alpha\beta$ secretion have been observed in humans (Schlaak et al.) it is within the realms of possibility that differences in an individual's type I IFN response could affect the outcome of IAV infection.

To investigate the contribution of IFN $\alpha\beta$ and in control of IAV replication and orchestration of the innate immune response to IAV I employed a variety of inbred laboratory mouse strains, associating susceptibility to IAV induced disease with IFN $\alpha\beta$ response. In order to ensure the phenomenon I am investigating is primarily dependent upon host response and not specific to aspects of the lab-adapted strain of IAV used, I used several strains of IAV, where appropriate, for broad profiling. Genetic modification, treatment of mice with mAb or exogenous administration of IFN α was used to modulate the level of IFN $\alpha\beta$ signalling in a given inbred mouse strain and assess the downstream effects of IFN $\alpha\beta$. From these results we find that IAV induced disease severity positively correlates with pulmonary concentrations of IFN $\alpha\beta$, and this is dependent upon the host intrinsic response, not elevated IAV titres. Alterations in number of functional alleles for the low affinity subunit of IFN $\alpha\beta$ R, IFNAR1, influenced IAV induced disease outcome. Significantly, triplication of a genomic region including IFNAR1 in resistant inbred mouse strain associated with increased IAV induced disease severity and higher pulmonary concentrations of IFN α .

Based upon the results attained in inbred mouse strains, I then went on to assess the viability of IFN α and IFN λ as a treatment for IAV infection. Isolation and

stimulation of specific immune cell types and epithelial cells allowed me to investigate cell type specific effects of IFN α and IFN λ . Analysis of viral titres, proinflammatory cytokine levels and inflammatory cell infiltrate in the lungs of IFN treated IAV infected mice demonstrated that exogenous IFN α treatment recapitulated severe disease features observed in high IFN $\alpha\beta$ producing strains. While the match of IFN λ R expression and IAV tissue tropism allows IFN λ s to target cell types at risk of infection, effectively inducing antiviral genes in these cells and therefore assisting in the control of IAV spread, without the risk of stimulating the immune system to enhance pathology.

Chapter 2. Materials and Methods

Mice

129S7/SvEvBrd-Hprt^b-m2 mice and IFN type I receptor α -chain-deficient (IFN $\alpha\beta$ R^{-/-}(129)) generated on the 129SvEv background (Muller et al., 1994) and their Wt control: 129SvEv (129) were purchased from B&K Universal. Identity of the genetic background between these strains was confirmed by SNP and microsatellite analysis (Charles River). IFN $\alpha\beta$ R^{-/-}(B6) mice, backcrossed for more than 10 generations to C57BL/6 mice were kindly provided by Dr. A. O'Garra (Francis Crick Institute) from Dr. M. Albert (Institut Pasteur, Paris). CBA/J mice were also kindly provided by Dr A. O'Garra. Recombination Activating Gene-2 deficient (Rag-2^{-/-}) (Shinkai et al., 1992) mice on the 129S6 background were kindly provided by Dr. F. Powrie (Univ. of Oxford). B6.A2G-Mx1 congenic mice carrying functional Mx1 alleles on the C57BL/6 background (Staeheli et al., 1985) (kind gift from Dr P. Staeheli, Freiburg Univ.). C57BL/6J.129P2-Dp(16Ifnar1-Runx1)^{8TybEmcf/Nimr} (Dp8Tyb) mice were kindly provided by Dr. Victor Tybulewicz (Francis Crick Institute). The above mice, 129S8, BALB/C, C57BL/6 (B6), Rag-1^{-/-} (B6) (Mombaerts et al., 1992), (129xB6)F1, IFNAR1 129 and IFNAR1 B6 mice were bred at the Francis Crick Institute, Mill Hill Laboratory under specific pathogen-free conditions. DBA/1 and 129X1/SvJ mice were purchased from Jackson Laboratory, DBA/2 mice from Harlan, 129S6/SvEv-Stat1^{tm1Rds} (Stat1^{-/-}), 129S5 and 129S6 from Taconic, and kept in specific pathogen-free isolators until use for experiments. Clinical symptoms during influenza infection were scored based upon presentation of piloerection, hunched posture, laboured breathing and reduced movement. All protocols for breeding and experiments with animals were approved by the Home Office, UK, Animals (Scientific Procedures) Act 1986.

Influenza Viruses

H3N2 A/Hong Kong/X31/68 (X31, a H3N2 reassortant with PR8 backbone), A/PR/8/34 (PR8, H1N1), and A/California/04/09 (Cal09, H1N1) (kind gifts from Dr. J. Skehel, Francis Crick Institute) were grown in the allantoic cavity of 10 day-embryonated hen's eggs and were free of bacterial, mycoplasma, and endotoxin contamination. Alternatively, virus was grown in Madin-Darby Canine Kidney (MDCK) cells. Egg allantoic cavity or MDCK cell supernatant was collected and ultracentrifuged to purify virus then stored at -70°C and titrated on MDCK cells. Mice were anaesthetised by inhalation with isofluorane and infected via the intranasal (i.n) route with 30µL of indicated influenza strain diluted in phosphate-buffered saline (PBS). Anaesthesia performed for experiments for Chapter 4 were done on animals kept on a heat mat to regulate body temperature. Virus was quantified in infected lungs by quantitative real-time PCR (qPCR) on complementary Deoxyribonucleic acid (cDNA) from whole lungs for the Matrix gene, as previously described (Ward et al., 2004) .

5'-AAGACCAATCCTGTCACCTCTGA-3'	PR8_M1 sense
5'-CAAAGCGTCTACGCTGCAGTCC-3'-	PR8_M1 antisense CR
FAM-5'-TTTGTGTTACGCTCACCGT-3'-TAMRA	PR8_M1 probe

Alternatively, IAV load in infected lungs was titrated on MDCK cells. Aliquots of whole lung samples prepared using gentleMACS tubes (see flow cytometry) was taken, centrifuged at 1,300 r.p.m., 5min at 4°C and supernatant collected, stored at -70°C until analysed. For samples prepared in Chapter 4: Whole lungs from infected mice were collected on ice. Lungs were minced and pressed through a 70µM strainer using 1ml of PBS. Samples were then centrifuged at 1,300 r.p.m., 5min at 4°C and supernatant collected, stored at -70°C until analysed. The

50% tissue culture infective dose (TCID₅₀) was determined by eight replicates of 10-fold serial dilutions using the Spearman and Faerber fit.

Treatment of Mice

To deplete 120G8⁺ cells, 129 mice were treated with α PDCA-1 (Cambridge Bioscience) or IgG2b isotype-matched control, 500 μ g/200 μ L i.p. on day 1 of infection with X31: 800 TCID₅₀, and every 48 h thereafter. 129 mice were treated with the Gr-1 reactive RB6-8C5, Ly6G reactive 1A8 or Isotype control (IgG2b) (500 μ g/200 μ L i.p.) (all from BioXCell) (on day 1 of infection and repeated every 48 h throughout X31 (800 TCID₅₀) infection. To deplete NK cells, 129 mice were treated with rabbit α Asialo GM1 serum (200 μ L) (eBioscience) on days 1, 3 and 7 post X31 (800 TCID₅₀) infection. To deplete CD8⁺ T cells, 129 mice were treated with mAb α CD8 (100 μ g/200 μ L) (eBioscience) one day prior to and at 5dpi with X31 (800 TCID₅₀). All depletions were confirmed by flow cytometry. To block TRAIL action, 129 mice were treated i.p. with 150 μ g/200 μ L of α CD253 (N2B2) (Cambridge Bioscience) or isotype control (IgG2a) every 24 h on days 0–9 post infection with 800 TCID₅₀ of X31. C57BL/6 mice were infected with X31 (8,000 TCID₅₀/30 μ L) or inoculated with Vehicle control i.n., then treated with Recombinant Mouse IFN α 4 (PBL Assay Science), 3.5x10⁴U/200 μ L or Vehicle Control via the intraperitoneal (i.p.) route on days 1–6 post infection (Chapter 3). B6.A2G-Mx mice were treated with 2x10⁵U/50 μ L of IFN α 4 or 2.6 μ g/50 μ L IFN λ 2 either at -1dpi (pretreatment experiment) or days 2, 4 and 5 post infection (treatment during infection experiments).

Recombinant IFN λ protein

A kind gift from Dr. Rune Hartmann. A codon optimized cDNA encoding the mature form (without the signal peptide) of Mouse IFN- λ 2 was purchased (Eurofins) and expressed in *E. Coli*, purified under denaturizing condition and refolded in vitro as described previously (Dellgren et al., 2009)

Bone Marrow Chimera generation

8 week old female 129 and IFN $\alpha\beta$ R^{-/-}(129) mice were given a split dose of lethal irradiation (500 rads x2 with an 8hr interval) the left to rest for 4hrs. During this time bone marrow from 8 week old female 129 and IFN $\alpha\beta$ R^{-/-}(129) mice was isolated. Briefly, legs were dissected from mice and bone marrow cells were obtained by crushing femurs and tibias with a mortar and pestle in RPMI-1640 (BioWhittaker). Red blood cells were lysed using ammonium chloride, and cells were washed x2 with MACS buffer (2% BSA, 2mM EDTA (PBS)). T and B cells were then depleted using QuadroMACS separator as per manufacture's instructions (Miltenyi Biotec). Briefly, CD8a⁺, CD4⁺ and CD19⁺ cells were magnetically labelled with MicroBeads (Miltenyi Biotec), for 10 minutes on ice. Binding was halted with the addition of excess MACS buffer and cells were centrifuged, 1300rpm, 4°C, 5 minutes. Cells were then washed once and loaded on a MACS® Column which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). Negatively selected cells were collected and the positive fraction discarded. Cells were then counted and washed x2 with PBS and after dilution to the appropriate density delivered via tail vein injection to irradiated mice. Irradiated mice were closely monitored after bone marrow transplant and kept on antibiotic (Baytril, Bayer)

supplemented drinking water for 4 weeks. Mice were left for a further 4 weeks before commencement of experiment.

RNA extraction

Whole lungs were collected in TRIzol (Invitrogen) and homogenized using Polytron PT 10-35 GT (Kinematica). AEC cultures were lysed directly in the transwells and immune cell cultures were lysed directly in wells using the Qiagen RNeasy mini kit, according to the manufacturer's instructions. Total RNA was prepared using phenol/chloroform extraction, and cDNA was generated from these samples using Thermoscript RT-PCR system, following manufacturer's instructions (Invitrogen). The cDNA served as a template for the amplification of genes of interest and the housekeeping gene (Hprt1) by real-time PCR, using TaqMan Gene Expression Assays (Applied Biosystems), universal PCR Master Mix (Applied Biosystems) and the ABI-PRISM 7900 sequence detection system (Applied Biosystems). The fold increase in mRNA expression was determined using the $\Delta\Delta C_t$ method relatively to the values in mock-treated samples, after normalization to Hprt1 gene expression.

Microarray data analysis

Lungs were homogenized in TRI Reagent (RiboPure kit, Ambion), and total RNA isolated according to manufacturer's instruction. RNA was hybridized to Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Raw data were processed using GeneSpring GX version 11.5 (Agilent Technologies). After background subtraction, each probe was attributed a flag to denote its signal intensity detection P-value. Flags were used to filter out probe sets that did not

result in a 'present' or 'marginal' call in at least 50% of the samples, in any one out of the experimental conditions. The signal intensity of each probe was first normalized on the median intensity of that probe across the control group and then represented as log2 fold change relative to the controls. For Figure 8: a two-way ANOVA (parameters: treatment and genotype) was performed to identify gene significantly differentially expressed relative to controls (\geq fourfold change; $P < 0.01$, Benjamini-Hochberg multiple test correction). For Figure 30: a 1-way ANOVA was performed to identify gene significantly differentially expressed relative to controls (≥ 1.5 fold change; $P < 0.01$, Benjamini-Hochberg multiple test correction) which were further analysed by K-means clustering. Microarray data has been deposited in Gene Expression Omnibus database under accession codes GSE55403 (Figure 8) or GSE70628 (Figure 30).

Protein Analysis

Bronchioalveolar lavage (BAL) fluid was recovered from naïve and infected mice, centrifuged at 1,300rpm, 5min at 4°C and supernatant collected. Stimulated pDC, cDC, macrophage and AEC supernatants were collected after 24hr stimulation. Concentrations of IFN α (all subtypes), β (PBL Biomedical Laboratories) and λ (R&D) were measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions. Concentrations of Eotaxin, G-CSF, IFN γ , MCP-1, IP-10, Mip-1 α , Mip-1 β and IL-4, IL-6, IL-9, IL-10, IL-12(p70), IL-12(p40) and IL-13 were assessed by Milliplex Map Kit (Millipore) as per manufacturer's instructions and read on a Luminex 100 (BioRad).

Microneutralisation Assay

Neutralising antibodies in serum were assessed by a microneutralisation assay. Briefly, serum samples were heat inactivated for 30 min at 56°C, diluted 1:100 then serially diluted 1:3 in duplicate in 96 well flat bottomed plates. Serum dilutions were preincubated with X31, 300TCID₅₀/well for 1hr at 37°C then added to MDCK cells and incubated for a further 22hrs at 37°C. After incubation cells were washed and fixed and neutralization capability was then assessed by staining for Fluorescein isothiocyanate (FITC)-conjugated influenza nucleoprotein (Oxoid) and detected with horseradish peroxidase-conjugated anti-FITC antibody (Roche). The reaction was then developed with tetramethylbenzidine substrate (eBioscience) for 15 min, stopped using H₂SO₄ and absorbance was read at 450 nm using Safire2 reader (Tecan).

Flow cytometry

Leukocytes from the lung were enumerated using flow cytometry. In brief, lungs were excised from naïve, infected and/or treated mice, digested with 20µg/ml Liberase TL (Roche) and 50µg/ml DNase 1 (30 minutes at 37°C) and homogenized using gentleMACS (Miltenyi), following the manufacturer's instructions. Lungs were then passed through a 70µM cell strainer and washed with FACS buffer (10% BSA in PBS Azide). In the case of lungs prepared for staining with TRAIL or IFNλR mAbs lungs were directly mashed through a 70µM cell strainer and washed with FACS buffer. Red blood cells were lysed using ammonium chloride and cells were seeded into a 96-well U-bottom plate at 1x10⁶/well. Cells were preincubated with anti-FcγRIII/II (Fc block) in FACS buffer prior to a 30 min incubation with one or more fluorochrome-labelled antibodies (Appendix, Table 1). Cells were then

washed with PBS x2 and stained with a secondary antibody and incubated for a further 20 minutes at 4°C (if appropriate). Cells were then washed with PBS x2 counter stained with LIVE/DEAD® Fixable Dead Cell Stain (Life Technologies) and, or 7AAD (Life Technologies) to enumerate apoptotic and dead cells (respectively). For intracellular staining cells were incubated in Fixation/Permeabilization buffer (Affymetrix eBioscience) for 20 minutes at room temperature, washed once with Permeabilization Buffer (Affymetrix eBioscience) and incubated for 30 minutes, 4°C with anti-NP/M-FITC antibody (Imagen Oxoid) in permeabilization buffer then washed twice. All samples were resuspended in PBS and analyzed using a LSR II or BD LSRFortessa X-20 (Becton Dickinson).

In-vitro stimulation of pDCs (Chapters 3 and 5)

129, IFN α β R $^{-/-}$ (129) and B6 bone marrow cells were obtained by flushing femurs and tibias with RPMI-1640 (BioWhittaker), using a 23 gauge needle. Red blood cells were lysed using ammonium chloride and cells were cultured in Flt3 supplemented (100ng/ml) (Pepro Tech) culture media (10% fetal calf serum (PAA), L-glutamine, penicillin, streptomycin, and β -mercaptoethanol in RPMI-1640). Media was replenished at day 4 of culture and cells were harvested at day 7. Harvested cells were preincubated with Fc block and biotin-conjugated B220 in 2% BSA (PBS) prior to 30 min incubation with anti-biotin conjugated magnetic beads. pDCs were then positively selected using an LS Columns and the QuadroMACS separator, as per manufactures instructions (Miltenyi Biotech) and found to be 90% pure based on FSC $^{\text{lo}}$, SSC $^{\text{lo}}$, PDCA-1 $^{+}$ and Siglec-H $^{+}$ as analysed by flow cytometry. For macrophages, culture media was supplemented with L cell sup (10%, kind gift from Anne O'Garra, FCI-MH). Media was replenished at day 4 of

cultures and harvested at day 7. Macrophages were isolated from culture by collection of the adherent cells. Culture was found to contain 95% macrophages, identified as FSC^{hi}, SSC^{hi}, F4/80⁺, CD11b⁺, by flow cytometry. pDCs were seeded at 6x10⁴ cells/well and macrophages at 2x10⁵ cells/well then rested for 24hrs prior to stimulation with MDCK grown X31 (MOI of 1) or vehicle control for 24hrs. Supernatants were then collected and stored at -70°C until samples were analysed.

In-vitro stimulation of pDCs, cDCs and Macrophages (Chapter 4)

B6 bone marrow cells were obtained by crushing femurs and tibias with a mortar and pestle in RPMI-1640 (BioWhittaker). Red blood cells were lysed using ammonium chloride, and cells were cultured in culture media (10% fetal calf serum (PAA), L-glutamine, penicillin, streptomycin, and β -mercaptoethanol in RPMI-1640) supplemented with Flt3L (100 ng/ml, Pepro Tech) for pDCs and cDCs or, for macrophages, supplemented with L cell sup (10%, kind gift from Anne O'Garra, FCI-MH) culture media. Media was replaced at day 4 of cultures and harvested at day 7. Macrophages were isolated from culture by collection of the adherent cells. Culture was found to contain 95% macrophages, identified as FSC^{hi}, SSC^{hi}, F4/80⁺, CD11b⁺ by flow cytometry. For pDCs and cDCs, non-adherent cells were collected and pre-incubated with Fc blocking mAbs and biotin-conjugated anti-B220 (Biolegend) in 2% FCS (PBS) before a 30-min incubation with anti-biotin conjugated magnetic beads. pDCs were then positively selected using an LS Columns and the QuadroMACS separator, following the manufacturer's instructions (Miltenyi Biotech), and found to be 95% pure based on FSC^{lo}, SSC^{lo}, PDCA-1⁺ and Siglec-H⁺ as analysed by flow cytometry. cDCs were collect from negative fraction and were found to be 90% pure based on FSC^{int}, SSC^{int}, CD11c⁺

and CD11b+. All cell types were seeded at 2×10^5 cells per well, and rested for 24 h before stimulation with IFN α 4 (100U/ml), IFN λ 2 (1.4ng/ml) or media controls for 24hrs. Supernatants were then collected and stored at -70°C until samples were analysed.

In-vitro stimulation of splenocytes

Spleens were excised from 129, IFNAR1+/- (129), IFN α β R-/- (129) and B6 mice. Spleens were then directly mashed through a 70 μ M cell strainer and washed with FACS buffer. Red blood cells were lysed using ammonium chloride and cells were seeded into a 96-well U-bottom plate at 1×10^6 /well. If stimulated whole splenocyte cultures were stimulated with IFN α 4 (100U/ml), IFN λ 2 (1.4ng/ml) or left as media control for specified time points. After stimulation cells were collected for analysis by flow cytometry.

Primary mouse tracheal epithelial cell culture

Isolation and culture of primary mouse tracheal epithelial cell culture were performed as described (Crotta et al., 2013): in brief, cells were isolated from mouse trachea by enzymatic treatment and seeded onto a 0.4 μ m pore size clear polyester membrane (Corning) coated with a collagen solution. At confluence, medium was removed from the upper chamber to establish an air-liquid interface (ALI). Fully differentiated, 7- to 10-day-old post-ALI cultures were routinely used for experiments. For ISG and IFN induction AECs were infected MDCK grown X31 (MOI of approx.. 1) (Chapter 3). For analysis of ISG induction and cytokine secretion, AEC cultures were stimulated with IFN α 4 (100U/ml) (PBL Assay Science), IFN λ 2 (1.4ng/ml) or medium control (Chapter 4).

Histology

Whole lungs were perfused with 10% neutral buffered formaldehyde (NBF) in situ. Tissue was then fixed overnight in 10% NBF, embedded in paraffin and sectioned. Each lung specimen was stained with hematoxylin and eosin (H&E) and then subjected to gross and microscopic pathologic analysis. For Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining, slides were deparaffinized and stained for apoptotic cells using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Miltenyi) as per the manufacturer's instructions. Imaging of slides was performed on a VS120 slide scanner (Olympus) with a VC50 camera, a UPLSAPO lens, at magnification of 20x and a numerical aperture of 0.75. Images were analysed using OlyVia Image Viewer 2.6 (Olympus) and Icy Spot Detector (ICY-R3M2Y2).

Statistical Analysis

Data shown as the means \pm SEM. Sample sizes were designed to give statistical power, while minimizing animal use. Data sets were analysed by 2-way ANOVA with Bonferroni post tests (weight, cytokine concentration and cellular recruitment time courses), Log-rank (Mantel-Cox) Test (survival) and Student t Tests (cytokine or gene induction from cells) or 2-way ANOVA (human samples). GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for data analysis and preparation of all graphs. P-values less than 0.05 were considered to be statistically significant.

Chapter 3. The pathogenic potential of IFN $\alpha\beta$ in IAV infection

Much of the experimental results in this chapter have been published in 'Pathogenic potential of interferon $\alpha\beta$ in acute influenza infection' Sophia Davidson, Stefania Crotta, Teresa M McCabe & Andreas Wack. 2014. Nature Communications 5, Article number: 3864 doi:10.1038/ncomms4864.

3.1 Background

Host susceptibility to, or protection from influenza is traditionally attributed to differences in virus strain virulence or varying degrees of pre-existing or newly acquired adaptive immunity of the host. Host genetics also play a role in the outcome of infectious disease (Alcais et al., 2010). A 2008 study conducted by Albright et al. examining the genealogy of a population from Utah, USA, linked to death certificates over a period of 100 years, observed that both close and distant relatives of individuals who died of influenza had a significantly increased risk of dying of influenza themselves, consistent with a combination of shared exposure and genetic effects (Albright et al., 2008). This study is particularly compelling as there was correlation between genetically related family groups rather than non-blood related family members (e.g.: spouses) within the same generation, indicating that similar exposure history may be less relevant than intrinsic host response. More generally, this last decade has seen the spread of avian influenza that, although highly virulent in avian species, has a relatively low rate of infection in humans. It has been observed that among the clusters of human infections with avian influenza, over 90% of cases have occurred in genetically related family groups (Horby et al., 2010; Olsen et al., 2005; Pitzer et al., 2007). Furthermore, several studies using outbred and inbred mice show a wide range of host-specific

genetic susceptibility with many candidate genes proposed to contribute to susceptibility or resistance (Alberts et al., 2010; Boon et al., 2009; Boon et al., 2011; Everitt et al., 2012; Haller et al., 1979; Srivastava et al., 2009). There is therefore a strong indication that variations in host genetics play a significant role in determining the susceptibility of an individual to influenza infection and subsequent disease outcome.

Although IAV was first characterised in 1933 (Smith, 1933), interactions between IAV and the immune response are yet to be fully elucidated. Specific anti-viral immune mechanisms which are known to contribute to IAV clearance and occur late in infection, such as induction of cytotoxic T lymphocytes (CTLs) and IAV specific antibodies (Eichelberger et al., 1991a; Eichelberger et al., 1991b; Epstein et al., 1998; Scherle et al., 1992; Topham et al., 1996), are well characterised, yet comparatively less is known about the early innate immune response. This is significant, as the innate response initially controls viral replication and directs the quality and magnitude of the adaptive immune response. In particular, some of the first cytokines to be released post influenza infection of airway epithelial cells are the type I IFNs (Crotta et al., 2013).

As both IFN α subtypes and IFN β exclusively signal through IFN $\alpha\beta$ R, many studies designed to elucidate the role of type I IFNs in influenza infection have been conducted under IFN $\alpha\beta$ R deficiency. In vitro studies have demonstrated IFN $\alpha\beta$ to be potent a inhibitor of IAV replication (Garcia-Sastre and Biron, 2006; Garcia-Sastre et al., 1998a; Isaacs and Lindenmann, 1957; Koerner et al., 2007; Matzinger et al., 2013). However, investigation of IFN $\alpha\beta$ R deficiency in restricting influenza infection in vivo is less clear some studies suggesting a protective role for IFN $\alpha\beta$ (Durbin et al., 2000; Garcia-Sastre et al., 1998a; Koerner et al., 2007;

Szretter et al., 2009), others finding no effect (Mordstein et al., 2008; Price et al., 2000). Differences in influenza strain or dose and use of either IFN $\alpha\beta$ R-/- or STAT1-/- mice as models of IFN signalling deficiency on B6, 129, CD1 or mixed mouse backgrounds make comparison between (and even within) these studies difficult. In particular, the use of STAT1-/- to specifically ablate type I IFN signalling is problematic. While STAT1 does act downstream of IFN $\alpha\beta$ R, it has also been demonstrated to act downstream of the receptors for types II and III IFNs as well as a number of other cytokines that have wide-ranging effects, such as IL-6, IL-10 and IL-27 (Casanova et al., 2012). Thus, the 'type I IFN' mediated protection claimed to be lost in studies that have used STAT1-/- mice as a model for type I IFN signalling is likely a result of a combined effect on a range of cytokines.

This discrepancy of the importance of IFN $\alpha\beta$ in protection against influenza-induced disease is reflected in the human data. Hayden et al. found that IFN α concentration in nasal washings was proportional to symptom severity in humans experimentally infected with seasonal IAV (Hayden et al., 1998), however this also correlated to viral load and could therefore be secondary to the available stimulus. Human mortality to infections with the highly pathogenic 1918 'Spanish Flu' strain and the H5N1 avian strains has been attributed to immunopathology and is associated with hypercytokinemia (de Jong et al., 2006; Kash et al., 2006; Mauad et al., 2010). IFN $\alpha\beta$ induce the secretion of other pro-inflammatory cytokines such as IP-10, IL-6 and IFN γ , and these cytokines are commonly found in high levels in the serum of critically ill IAV infected humans (Agrati et al., 2010; Arankalle et al., 2010; Hayden et al., 1998; Kaiser et al., 2001; Peiris et al., 2004). Although in many of these studies IFN $\alpha\beta$ levels in the serum was tested it was rarely detected. However, while accessible from severely ill patients, serum is not

necessarily reflective of events occurring at the site of infection, and it is possible IFN $\alpha\beta$ induced secretion of proinflammatory cytokines in the lung is upstream of this 'spill-over' which is observed in the blood. Cheung et al. have demonstrated that human macrophages secrete high levels of IFN β within hours of H5N1 infection in vitro (Cheung et al., 2002), while other studies suggest that type I IFN levels are lower in H5N1 infection as compared to seasonal strains (Zeng et al., 2007). Unlike H5N1, H1N1 S-OIV mortality in humans has not been associated with hypercytokinemia. The generally low pathogenicity of H1N1 S-OIV (California strain) correlates well with a moderate induction of IFN $\alpha\beta$ (Woo et al., 2010). In contrast, severe disease due to the highly pathogenic 1918 strain of H1N1 in macaques is correlated with persisting low levels of IFN $\alpha\beta$ (Kobasa et al., 2007). Studies conducted in mouse models have not yet identified any specific immune features that drives the cytokine storm in IAV. However, Teijero et al. did demonstrate that IFN $\alpha\beta$ induced secretion of proinflammatory cytokines from endothelial cells and this contributed to host susceptibility to IAV (Teijero et al., 2011). In a backcrossing study between a resistant (C57BL/6) and a susceptible (DBA/1) mouse strain, Boon et al. observed that pulmonary concentrations of IFN $\alpha\beta$ during IAV infection positively correlated with disease severity (Boon et al., 2009), but no causal relationship was established.

The role of type I IFNs during IAV infection is therefore, highly controversial. It remains to be elucidated whether this cytokine drives not only an antiviral response but also promotes pathogenicity during IAV infection. Furthermore, it is unknown whether type I IFNs contribute to inter-individual differences in IAV susceptibility.

3.2 Hypothesis and Aims

This project aimed to investigate the role of type I IFN in influenza-induced disease. Using different inbred laboratory mouse strains, we correlated type I IFN response with disease outcome. By comparing mouse strains with high IFN and low IFN responses and their congenic strains deficient for IFN $\alpha\beta$ R, we were able to assess the importance of type I IFNs in induction of antiviral genes and immunomodulation during IAV infection.

I hypothesised:

- IFN $\alpha\beta$ can have a positive and negative effect on IAV induced disease outcome, depending on signal strength.
- IFN $\alpha\beta$ and IFN λ are redundant for induction of antiviral genes in airway epithelial cells.
- pDCs can be a major source of type I IFN during IAV infection.
- High amounts of type I IFN signalling during IAV infection can lead to over activation of the immune response.

3.3 Results

3.3.1 IFN $\alpha\beta$ and λ levels positively correlate with influenza-induced morbidity and mortality across different mouse strains

Comparison between the two inbred laboratory mouse strains C57BL/6 (B6) and 129S7/SvEvBrd-Hprt^b-m2 (129) mice led us to observe dramatic differences in morbidity and mortality during infection with the low pathogenicity H3N2 IAV strain, X31. B6 mice were highly resistant to X31 induced disease, over several logs of viral inoculate. In contrast, 129 mice developed pronounced clinical symptoms such as piloerection, reduced movement, laboured breathing and dramatic weight loss, ultimately resulting in host mortality (Figure 2A-B). Increased morbidity of the 129 mice associated with tissue damage and a robust pro-inflammatory response (Figures 5-7). All other 129 substrains tested, i.e. 129 X1/SvJ, S5, S6 and S8, also exhibited high susceptibility to X31 induced disease compared to B6 mice (Figure 2C). Furthermore, infection with the pandemic H1N1 IAV strain Cal09 and the classical H1N1 strain PR8 also demonstrated increased susceptibility of 129 mice to influenza-induced disease (Figure 2D).

Intriguingly, concentrations of the antiviral cytokines IFN α , β and λ in the lungs of susceptible 129 mice were markedly higher than concentrations observed in B6 lungs at the majority of time points tested post X31 infection, as assessed in bronchoalveolar lavage (BAL) fluid (Figure 3A) and whole lung homogenates (not shown). As greater viral replication in 129 mice may stimulate higher expression of IFN $\alpha\beta$, we measured copy number of X31 matrix by qPCR between 1 and 48hrs post infection, a period where differences in IFN $\alpha\beta$ expression between 129 and B6 mice is already established. Presence of X31 matrix in 129 and B6 lungs was not

Figure 2

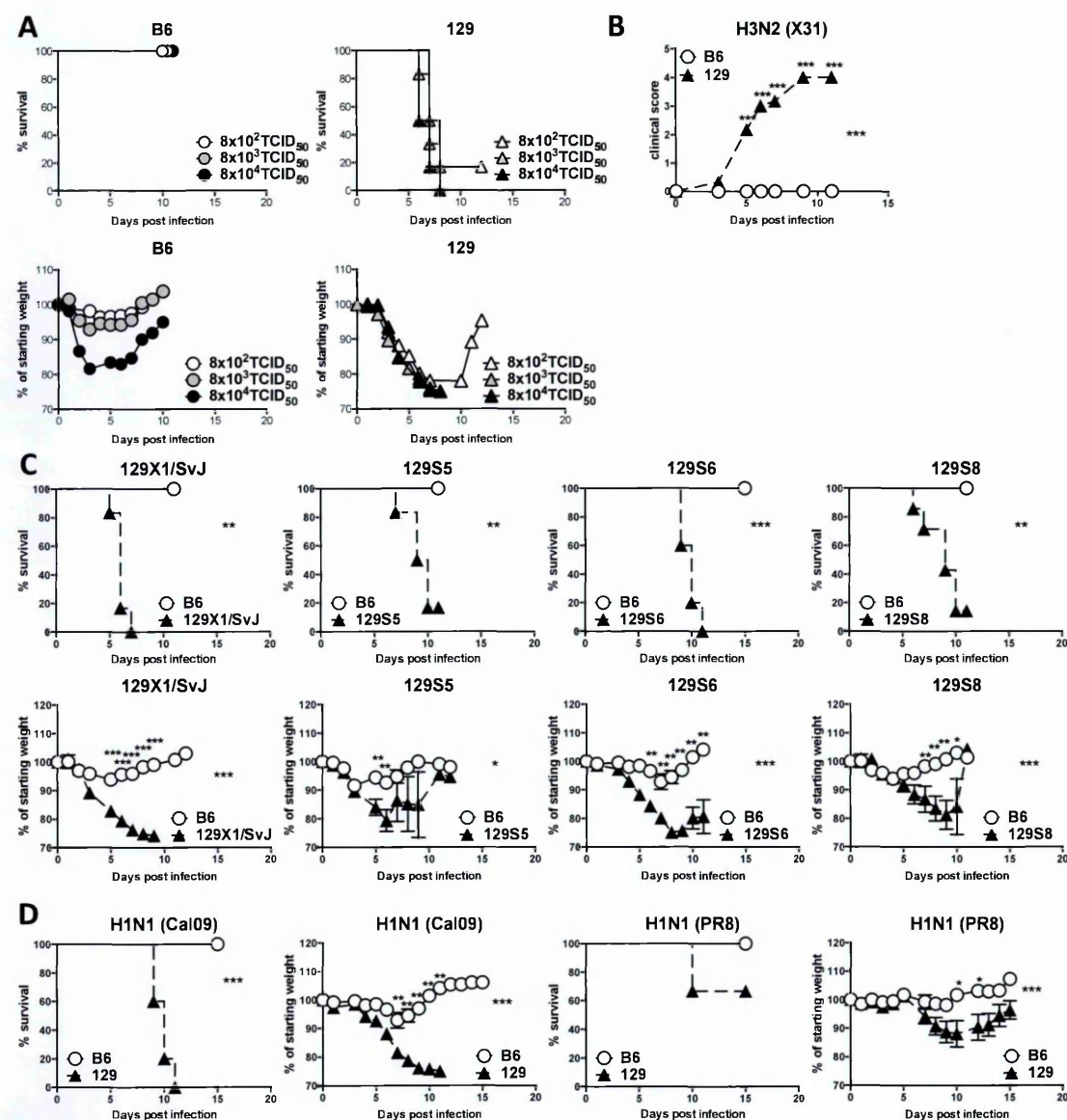


Figure 2: 129 mice are more susceptible than B6 mice to IAV induced disease across a range of virus strains and doses. (A) B6 (circles) and 129 (triangles) mice were infected i.n. with IAV strain: X31 at indicated viral doses and weight loss and mortality recorded. (B) 129 and B6 mice were infected with X31 (800 TCID₅₀) and scored based on presentation of clinical symptoms. (C) Indicated 129 substrains (triangles) and B6 mice were infected (i.n.) with X31 (800 TCID₅₀) and weight loss and mortality recorded. (D) 129 and B6 mice were infected i.n. by indicated influenza virus strains: Cal09 (1000 TCID₅₀) or PR8 (5 TCID₅₀), and weight loss and mortality recorded. Graphs show mean \pm s.e.m and are representative of 2-5 independent experiments where $n \geq 6$. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$ by 2-way ANOVA with Bonferroni post tests (weight loss or clinical score) or Log-rank (Mantel-Cox) Test (survival). Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 3

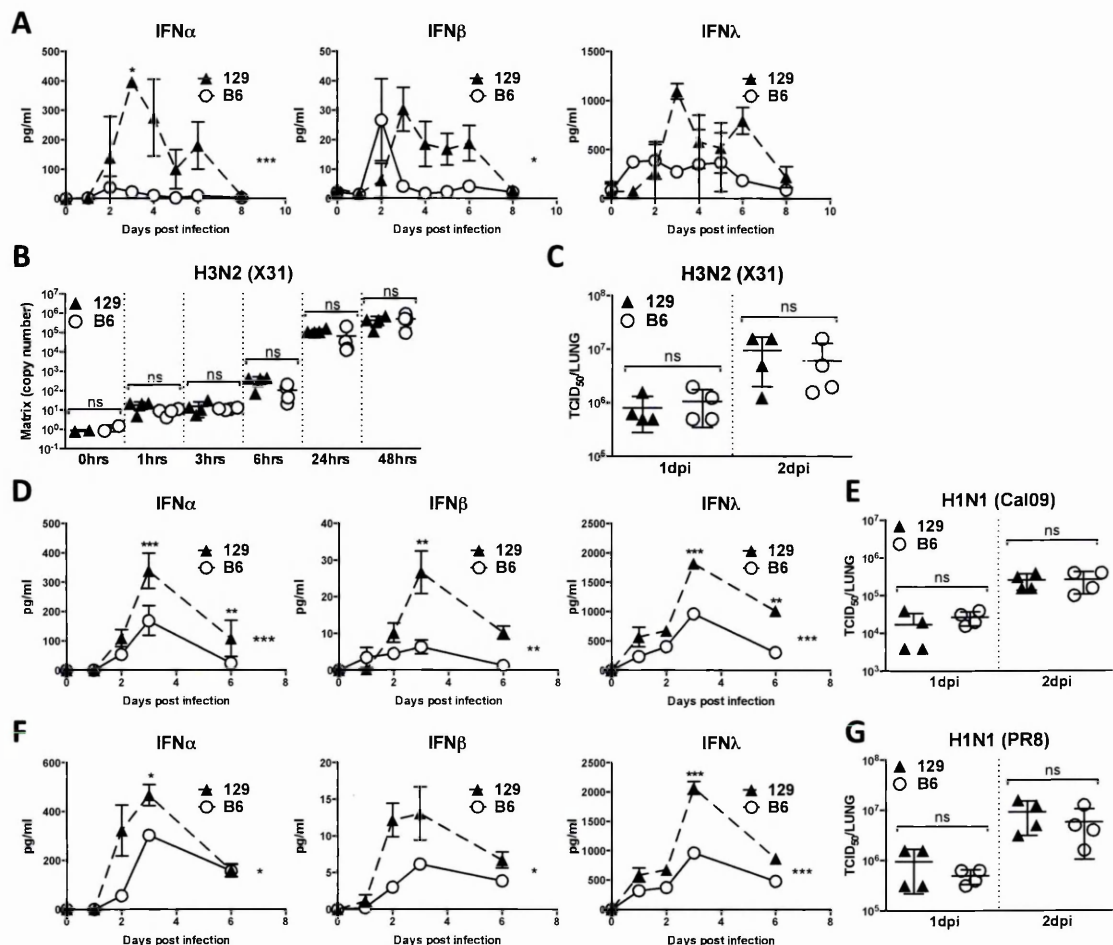


Figure 3: Increased susceptibility of 129 mice to IAV-induced disease correlates with higher BAL concentrations of IFN α , β and λ , despite comparable viral load. B6 (circles) and 129 (triangles) mice were infected with X31 (800 TCID₅₀) i.n. and (A) IFN α , β and λ levels in BAL fluid were measured by ELISA. X31 burden was quantitated by (B) qPCR for the X31 Matrix gene on cDNA from whole lungs and (C) by titration of whole lungs on MDCK cells for TCID₅₀ determination. (D-E) 129 and B6 mice were infected with Cal09 (1,000 TCID₅₀), (D) concentration of IFN α , β and λ in the BAL fluid was assessed by ELISA and (E) Cal09 TCID₅₀ was assessed by titration of whole lung homogenates. (F-G) 129 and B6 mice were infected with PR8 (100TCID₅₀). (F) concentration of IFN α , β and λ in the BAL fluid was assessed by ELISA and (F) PR8 lung titre was assessed by titration. Graphs show mean \pm s.e.m and are representative of 2-3 independent experiments where $n=3-4$. *** $p<0.0001$, ** $p<0.001$, * $p<0.01$ by 2-way ANOVA with Bonferroni post tests (ELISA) or Mann Whitney test (viral quantification). Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

statistically different at any early time point measured (Figure 3B). However, copy number is not indicative of infectious particles, therefore we also confirmed this result by titrating infected lung homogenates from 129 and B6 mice on MDCK cells. Viral titres, as assessed by TCID₅₀ in 129 and B6 lungs, were not significantly different at days 1 and 2 post infection (Figure 3C). Furthermore, assessment of IFN concentrations in the BAL and lung viral titres during Cal09 and PR8 infection confirmed higher IFN α , β and λ concentrations in 129 lungs and this is not due to a higher viral load compared to B6 lungs specific to one IAV strain (Figure 3D-G). We therefore conclude that differences in expression of type I IFNs is host intrinsic.

To extend the study we infected four other inbred mouse strains, BALB/C, CBA/J, DBA/1 and DBA/2, with X31 and assessed IFN $\alpha\beta$ and IFN λ levels in the lung and susceptibility to X31. Using B6 and 129 parameters as reference, we observed that like the B6 strain, BALB/C mice were more resistant to X31 induced disease and had low concentrations of IFN α , β and λ in their BAL at early time points of infection (Figure 4A, B). In contrast, CBA/J, DBA/1 and DBA/2 mice all exhibited marked weight loss and mortality during X31 infection (Figure 4C, E, G), and this associated with elevated concentrations of IFN α and β in BAL fluid (Figure 4D, F, H). As with 129 mice, overt type I IFN signalling in the pulmonary environment during X31 infection associated with increased host susceptibility. IFN λ levels trended to be higher in the susceptible strains than non-susceptible strains, however this was generally not significant. We can conclude from this series of experiments that high pulmonary IFN $\alpha\beta$ levels correlate directly, not inversely with influenza-induced morbidity and mortality across a wide range of mouse genetic backgrounds and virus strains.

Figure 4

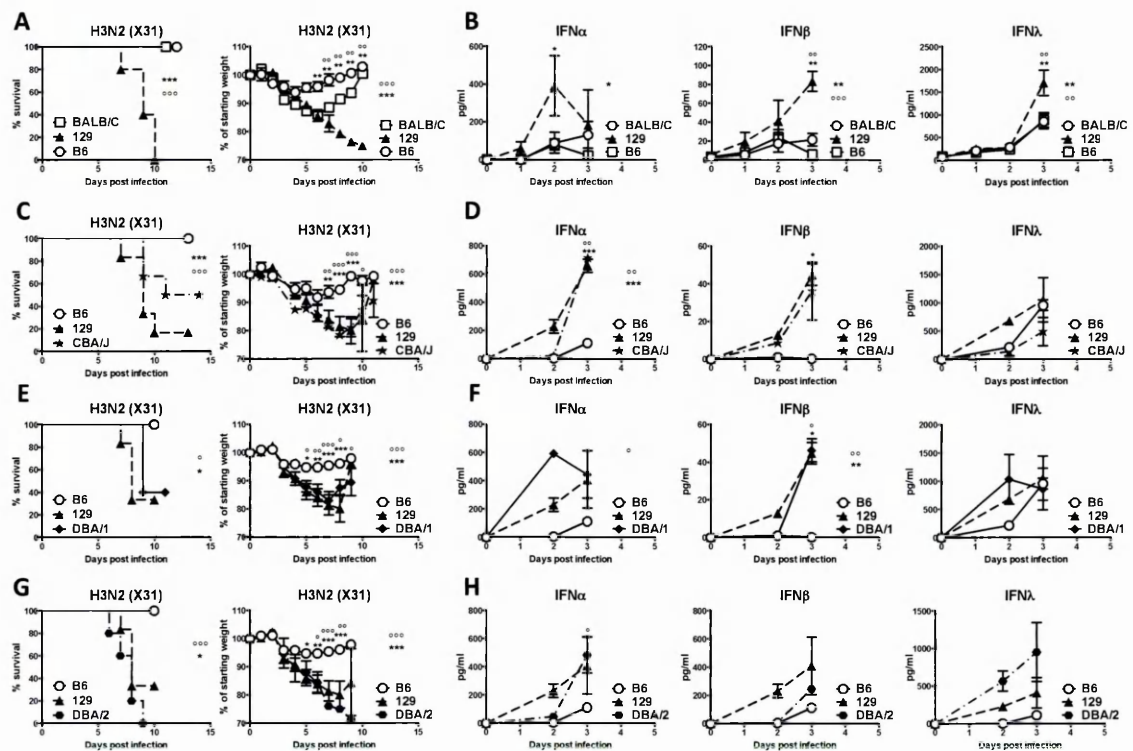


Figure 4: High susceptibility to IAV induced disease correlates with high pulmonary concentrations of IFN α , β and λ across a range of mouse strains. (A-B) B6 (circles), 129 (triangles) and BALB/C (open squares) mice were infected with X31 (8000 TCID₅₀), (A) weight loss and mortality recorded and (B) IFN concentrations in BAL were measured by ELISA. (C-H) 129, B6, CBA/J (black stars), DBA/1 (black diamonds) and DBA/2 (black ovals) mice were infected with X31 (800 TCID₅₀), (C, E, G) weight loss and mortality recorded and (D, F, H) IFN levels in BAL were measured by ELISA. Graphs show mean \pm s.e.m and are representative of 2-3 independent experiments where $n=3-6$. *** $p<0.0001$, ** $p<0.001$, * $p<0.01$ by 2-way ANOVA with Bonferroni post tests (weight loss and ELISA) or Log-rank (Mantel-Cox) Test (survival) where 129:B6 is denoted by *, 129:BALB/C by °, B6:CBA/J by °, B6:DBA/1 by ° and B6:DBA/2 by °. B6:BALB/C and 129:CBA/j, 120:DBA/1 and 129:DBA/2 were not significant. Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

3.3.2 IFN $\alpha\beta$ mediates influenza-induced host morbidity and mortality, pro-inflammatory cytokine secretion and cellular recruitment

Given the association between high levels of IFN $\alpha\beta$ and high susceptibility of 129 mice, we decided to investigate the effect of IFN α and β signalling in these mice. Since all IFN α and β molecules exclusively signal through the IFN $\alpha\beta$ R, we compared susceptibility of 129 mice deficient for this receptor to their wild type (Wt) controls. Surprisingly, ablation of IFN $\alpha\beta$ R signalling resulted in an increase in resistance to IAV induced disease. We observed significantly less weight loss and mortality in IFN $\alpha\beta$ R $^{-/-}$ (129) mice than Wt 129 mice during infection with X31, Cal09 or PR8 (Figure 5A). IFN $\alpha\beta$ deficiency lowered pulmonary concentrations of IFN α , β and λ throughout X31 infection, indeed levels of IFNs detected in IFN $\alpha\beta$ R $^{-/-}$ (129) samples were comparable to those found in B6 rather than Wt 129 BAL (Figure 5B). This indicates that IFN $\alpha\beta$ R signalling is required to maintain the high levels of both IFN $\alpha\beta$ and IFN λ that we observe in the 129 background.

Disease features observed in the 129 strain recapitulated cardinal markers of severe influenza induced disease in humans. Specifically, gross pathology analysis of whole lungs stained with hemolysin and eosin (H&E) revealed immense innate cell infiltrate and lung tissue damage in 129 lungs. Lower concentrations of IFN $\alpha\beta$, as in the B6 lung, or complete ablation of type I IFN signalling, as in IFN $\alpha\beta$ R $^{-/-}$ (129) lungs, lead to markedly less cellular infiltrate and better preservation of the alveolar structure during X31 infection (Figure 5C). Furthermore, 129 lungs exhibited increased concentrations of a number of pro-inflammatory cytokines including MCP-1, IL-6, IP-10, IFN γ , Eotaxin, G-CSF, and Mip-1 β , as compared to the resistant B6 strain. Secretion of these pro-inflammatory cytokines was also significantly reduced when IFN $\alpha\beta$ signalling was

Figure 5

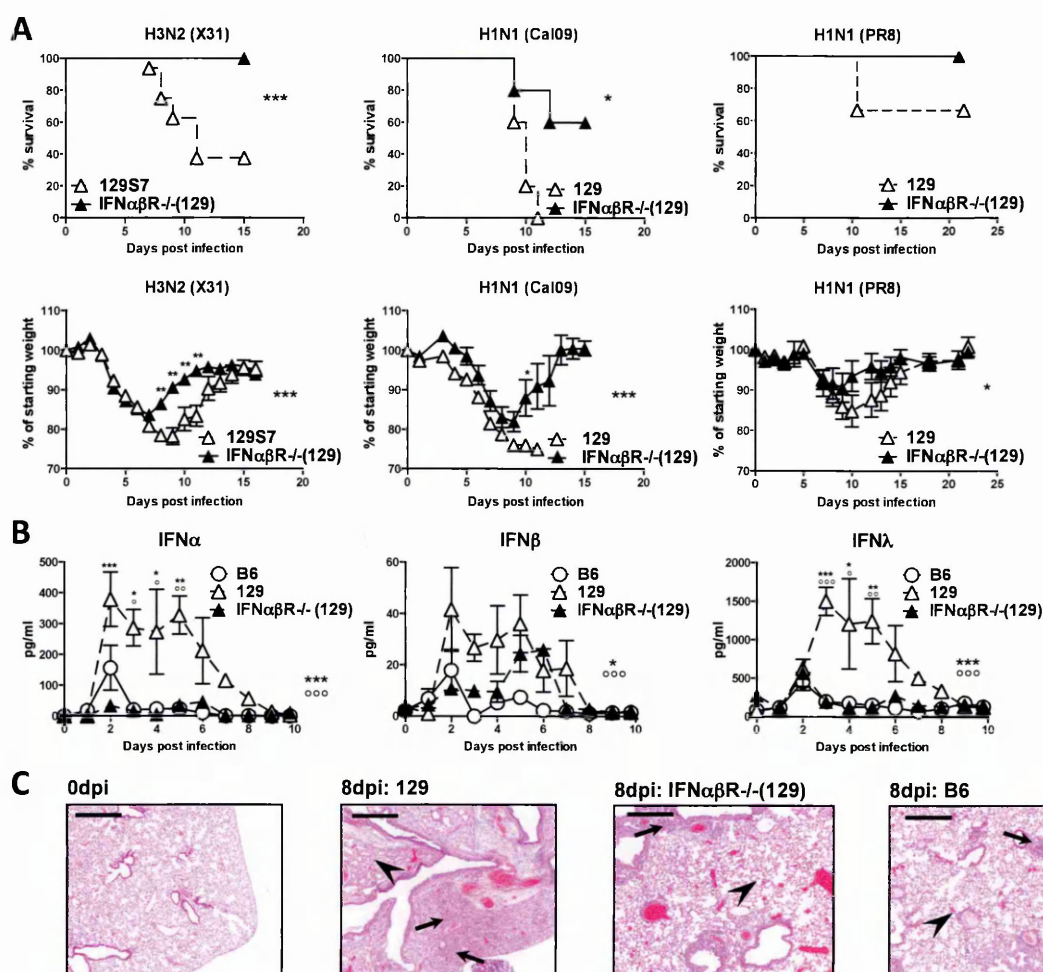


Figure 5: Genetic ablation of IFN $\alpha\beta$ signalling protects 129 mice from severe IAV induced disease. 129 (open triangles) or IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) mice were infected i.n. with X31 (800 TCID₅₀), Cal09 (330 TCID₅₀) or PR8 (5TCID₅₀). (A) Mortality and weight loss was recorded. (B) Concentration of IFN α , β and λ in the BAL fluid of X31 infected 129, IFN $\alpha\beta$ R $^{-/-}$ (129) and B6 mice were assessed by ELISA. (C) Whole lung sections were taken from uninfected and at 8 days post X31 infection from 129, IFN $\alpha\beta$ R $^{-/-}$ (129) and B6 mice and sections were stained with Haematoxylin & Eosin (H&E). Scale bar shows 50 μ m, arrows indicate leukocyte infiltrate and arrowheads indicate intact alveolar structure. Graphs show mean \pm s.e.m and are representative of 2-4 independent experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss and IFN time courses) or Log-rank (Mantel-Cox) Test (survival), where * 129:IFN $\alpha\beta$ R $^{-/-}$ (129) and ° denotes 129:B6. Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 6

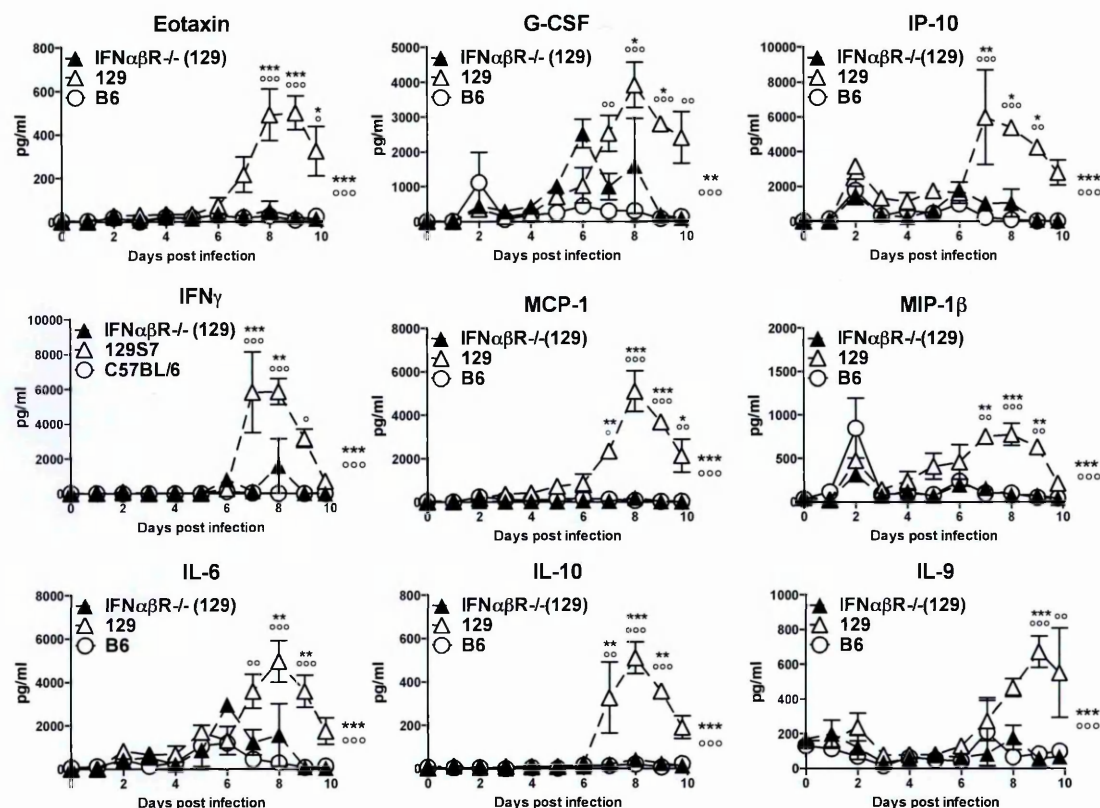


Figure 6: IFN $\alpha\beta$ signaling in 129 mice is upstream of high pulmonary concentrations of proinflammatory cytokines. 129 (open triangles), IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) and B6 (open circles) mice were infected with X31 (800TCID $_{50}$). Specified cytokine and chemokine concentrations were quantified by multiplex. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=3-4. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests where * indicates 129:IFN $\alpha\beta$ R $^{-/-}$ (129) and ° denotes 129:B6. Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

abolished in the 129 strain. Perhaps due to this massive inflammatory response, 129 lungs also exhibited elevated levels of IL-10 and IL-9, which dampen the immune response and drive tissue repair (respectively) (Figure 6).

Total cell counts from lungs infected with X31 indicated that although cell number does increase throughout infection, this is comparable between all mouse strains tested (Figure 7A). However, flow cytometric analysis of different cell types recruited during infection revealed higher numbers of pDCs (120G8⁺, Siglec H⁺, CD11c^{int}, CD11b⁻, FSC^{lo}, SSC^{lo}), IMcs (Ly6C^{hi}, CD11b⁺, CD11c⁻, FSC^{int}, SSC^{lo-int}) and NK cells (Nkp46⁺, CD3⁻, FSC^{lo}, SSC^{lo}) in 129 mice compared to both IFN $\alpha\beta$ R^{-/-}(129) and B6 mice (Figure 7B). Conversely, B cells (CD19⁺, CD3⁻, FSC^{lo}, SSC^{lo}) were found in higher numbers in B6 and IFN $\alpha\beta$ R^{-/-}(129) lungs late in infection, but not in 129 lungs (Figure 7C). Recruitment of Neutrophils (Ly6G⁺, CD11b⁺, CD11c⁻, FSC^{int}, SSC^{int-hi}) was comparable between 129 and B6 lungs, but increased in IFN $\alpha\beta$ R^{-/-}(129) lungs (Figure 7D). mDC (MHCII⁺, CD11b⁺, CD11c⁺, FSC^{int}, SSC^{lo-int}), CD4⁺ T cell (CD4⁺, CD8⁻, CD3⁺, FSC^{lo}, SSC^{lo}) and CD8⁺ T cell (CD8⁺, CD4⁻, CD3⁺, FSC^{lo}, SSC^{lo}) numbers were similar across all strains tested (Figure 7E). Blunted B cell recruitment in 129 lungs associated with a trend for lower X31 specific antibodies in the sera at 9dpi, as assessed by microneutralisation (Figure 7F).

3.3.3 IFN $\alpha\beta$ is dispensable for antiviral gene induction in IAV infected airway epithelia

Ablation of IFN $\alpha\beta$ signalling results in a lowering of the levels of proinflammatory cytokines in the BAL and decreased inflammatory cell recruitment, which correlates with increased resistance to influenza-induced pathology. Given the well-characterised antiviral properties of IFN $\alpha\beta$ it is

Figure 7

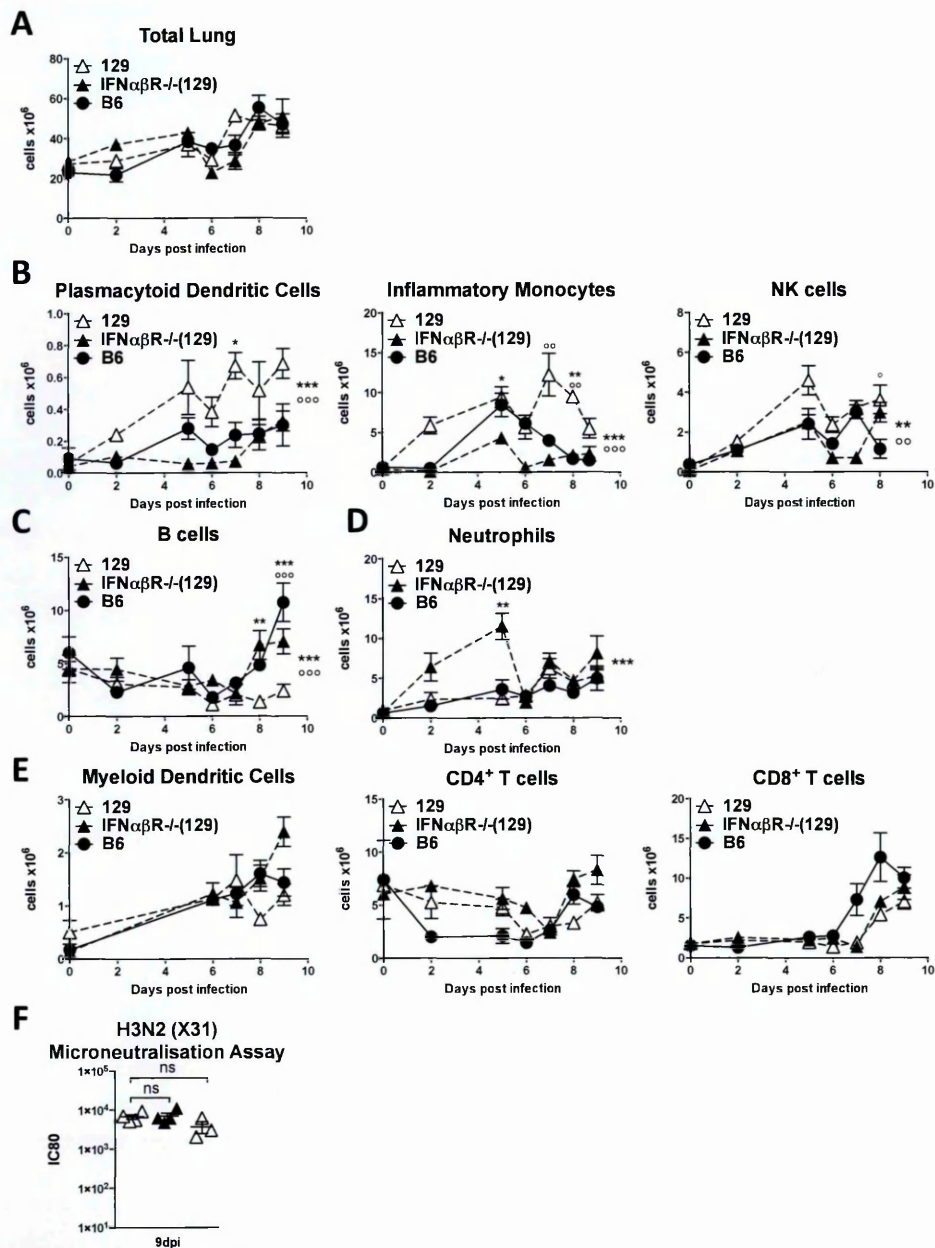


Figure 7: pDC, IMc and NK cells are preferentially recruited in X31 infected 129 lungs. 129 (open triangles), IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) and B6 (open circles) mice were infected with X31 (800TCID₅₀) and (A) total cell count of whole lungs was assessed at specified time points. (B-E) Flow cytometric quantification of pDCs, NK cells, IMs, B cells, Neutrophils, mDCs, CD4 $^{+}$ T cell and CD8 $^{+}$ T cell was performed. (F) At 9dpi sera was taken and X31 neutralizing antibody titre was measured by microneutralisation assay. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where $n=3-4$. *** $p<0.0001$, ** $p<0.001$, * $p<0.01$ by 2-way ANOVA with Bonferroni post tests (recruitment) or by Mann Whitney test (antibody titre) where * 129:IFN $\alpha\beta$ R $^{-/-}$ (129) and $^{\circ}$ denotes 129:B6. Symbols on the right of graphs indicate statistical significance of the whole curve, as assessed by 2 way ANOVA, symbols above individual points indicate significance as assessed by Bonferroni post test.

surprising that ablation of IFN $\alpha\beta$ signalling is protective. Indeed IFN $\alpha\beta$ R $^{-/-}$ (129) mice are able to control and clear X31 infection. Titration of whole lung homogenates from X31 infected 129 and IFN $\alpha\beta$ R $^{-/-}$ (129) mice revealed that virus clearance was comparable between the two strains in fact, IFN $\alpha\beta$ R $^{-/-}$ (129) controlled X31 viral load as well as, if not slightly better than their Wt counterparts, however this was not statistically significant (Figure 8A).

To investigate whether or not IFN $\alpha\beta$ R deficiency negatively impacted induction of antiviral ISGs we performed a microarray on whole lung samples taken at 5dpi. Induction of selected ISGs in infected lungs was comparable between B6, 129 and IFN $\alpha\beta$ R $^{-/-}$ (129) lungs (Figure 8B). Infection of airway epithelial cell (AEC) cultures also confirmed IFN $\alpha\beta$ R independent upregulation of common ISGs including IRF9, ifi203, Oasl2 and STAT2 (Figure 9A). Additionally, IFN $\alpha\beta$ R deficiency did not enhance X31 replication in these cultures (Figure 9B). These results are consistent with studies suggesting redundancy between IFN $\alpha\beta$ and IFN λ in airway epithelia. Interestingly, unlike what was observed in the whole mouse, IFN $\alpha\beta$ R deficiency does not alter IFN λ expression in AECs (Figure 9C), indicating that decreased levels of IFN λ recorded in the whole IFN $\alpha\beta$ R $^{-/-}$ (129) lung during X31 infection is a phenomenon specific to immune cells.

STAT1 is a signalling molecule essential for signal transduction of many proinflammatory cytokines, including all types of IFN. In contrast to IFN $\alpha\beta$ R $^{-/-}$ (129) AECs, STAT1 $^{-/-}$ (129) AECs were unable to upregulate ISGs upon influenza infection (Figure 9A). Yet STAT1 deficiency did not affect expression of IFN λ , which was comparable in 129, IFN $\alpha\beta$ R $^{-/-}$ (129) and STAT1 $^{-/-}$ (129) AECs (Figure 9C). The lack of ISG induction seen in STAT1 $^{-/-}$ (129) AECs correlated with significantly

Figure 8

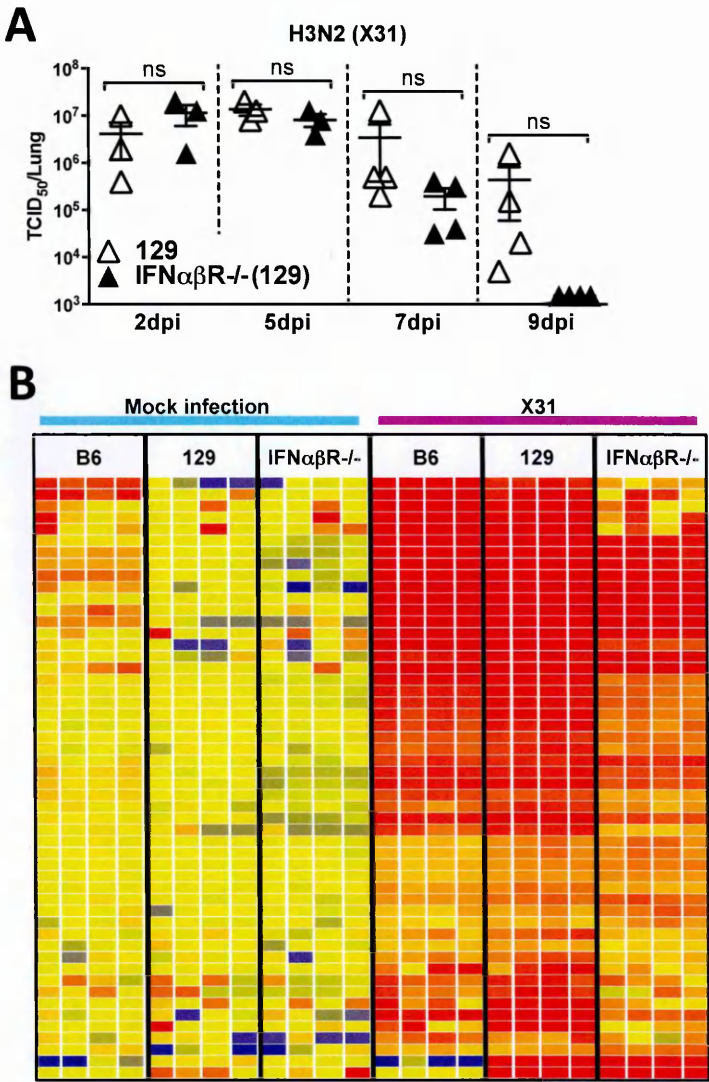


Figure 8: IFN $\alpha\beta$ R deficiency does not impede control X31 viral load or ISG induction. 129 (open triangles) and IFN $\alpha\beta$ R^{-/-}(129) (black triangles) mice were infected with X31 (800TCID₅₀) and (A) Viral titers in lung homogenates taken at the indicated time points were measured by TCID₅₀ determination on MDCK cells. (B) Heatmap displaying selected significantly regulated antiviral response genes. Total RNA from mock and X31-infected lung was analyzed using Affymetrix Mouse Genome 430 2.0 microarrays at 5 days post infection. Supervised analysis was performed using statistical filtering (\geq fourfold change relative to mock-infected C57BL/6; 2-way ANOVA, $P < 0.01$, Benjamini-Hochberg multiple test correction). X31 viral load significance was assessed by Mann Whitney test. Graphs show mean \pm s.e.m. and are representative of two independent experiments where n.3–4.

Figure 9

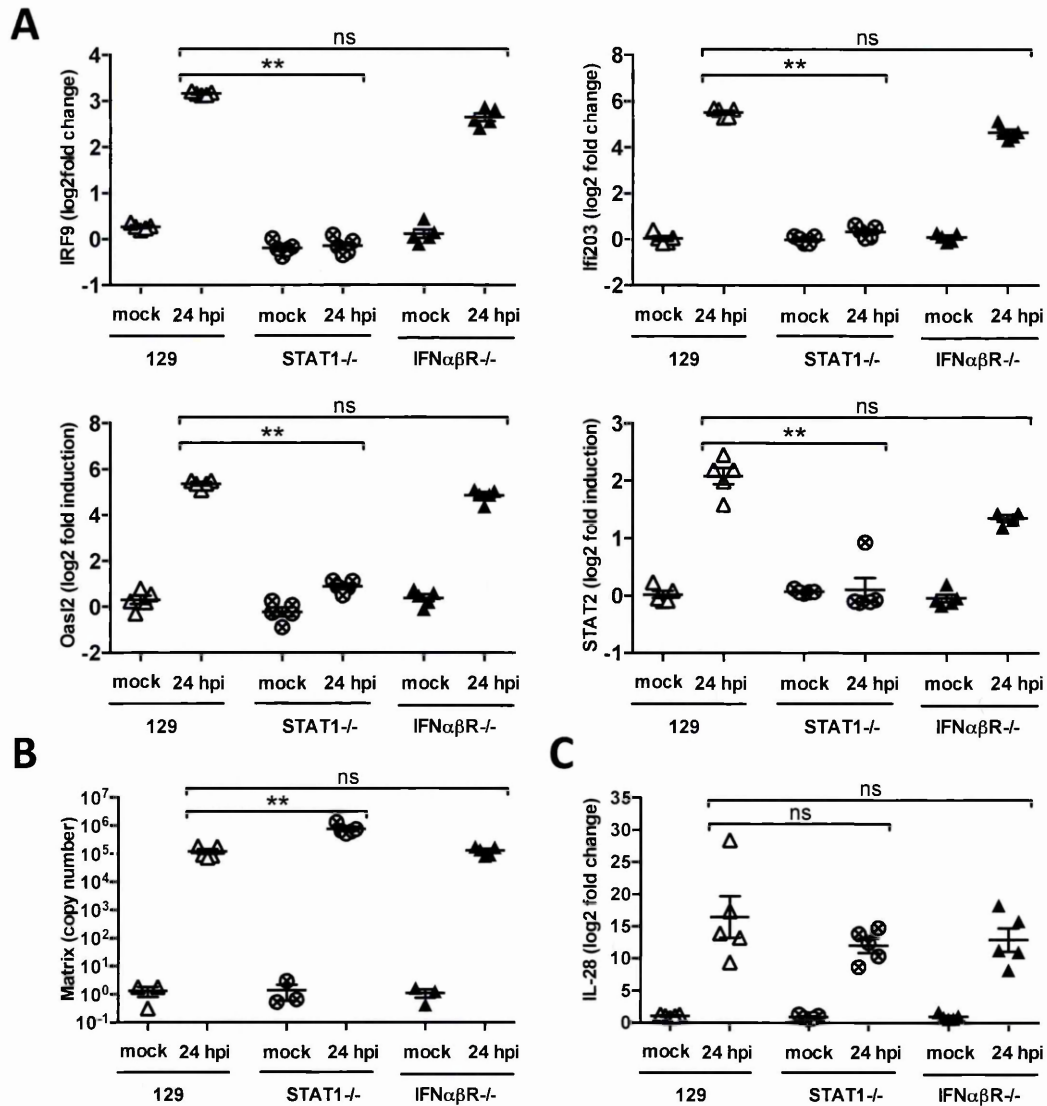


Figure 9: STAT1 but not IFNαβR is required for ISG induction and control of X31 replication in AECs. (A-C) 129 (open triangles), IFNαβR^{-/-}(129) (black triangles) and STAT1^{-/-}(129) (crossed circles) AEC cultures were infected with X31 at a MOI of 1. At 24 h post infection upregulation of (A) Oas12, STAT2, IRF9, Ifi203, (C) IL-28 (IFNλ) mRNA and (B) X31 Matrix was assessed by qPCR. Graphs show mean ± s.e.m. and are representative of two independent experiments where n=5. ** indicates P<0.001, as assessed by Mann-Whitney test.

higher X31 replication (Figure 9B). Furthermore, STAT1^{-/-}(129) mice were exquisitely susceptible to X31 induced disease, compared to both Wt and IFN $\alpha\beta$ R^{-/-} 129 mice (Figure 10A). Yet, in contrast to Wt 129 mice we did not observe a massive inflammatory response in STAT1^{-/-}(129) lungs. Indeed, with the exception of neutrophils, recruitment of all cell types assessed was largely dependent on STAT1 during X31 infection (Figure 10B). Additionally, STAT1^{-/-}(129) mice also experienced higher viral loads compared to Wt controls (Figure 10C). As STAT1 is essential for both IFN $\alpha\beta$ and IFN λ signalling, this inability to induce ISGs with antiviral function upon IAV infection in vivo leading to a permissive environment for IAV replication may explain the high susceptibility of these mice. Furthermore, these results indicate that some STAT1 dependent signalling is required for inflammation, which if controlled may be protective.

3.3.4 IFN $\alpha\beta$ mediated resistance to IAV-induced disease is a function of concentration

Our results indicate that in IAV infection IFN $\alpha\beta$ is upstream of inflammation, and depending on the magnitude of IFN $\alpha\beta$ signalling this can be protective or detrimental to the host. To understand whether or not this is specific to the 129 strain, we sought to ablate or augment IFN $\alpha\beta$ signalling in our resistant strain (B6 mice). Genetic ablation of the IFN $\alpha\beta$ R in B6 mice lead to an increase in host morbidity during both X31 and Cal09 infection yet ultimately, did not significantly alter mortality (Figure 11A). As was observed in IFN $\alpha\beta$ R^{-/-}(129) mice, recruitment of pDCs and IMcs was blunted, while neutrophil infiltration was higher in IFN $\alpha\beta$ R^{-/-}(B6) lungs, compared to their Wt controls. Recruitment of

Figure 10

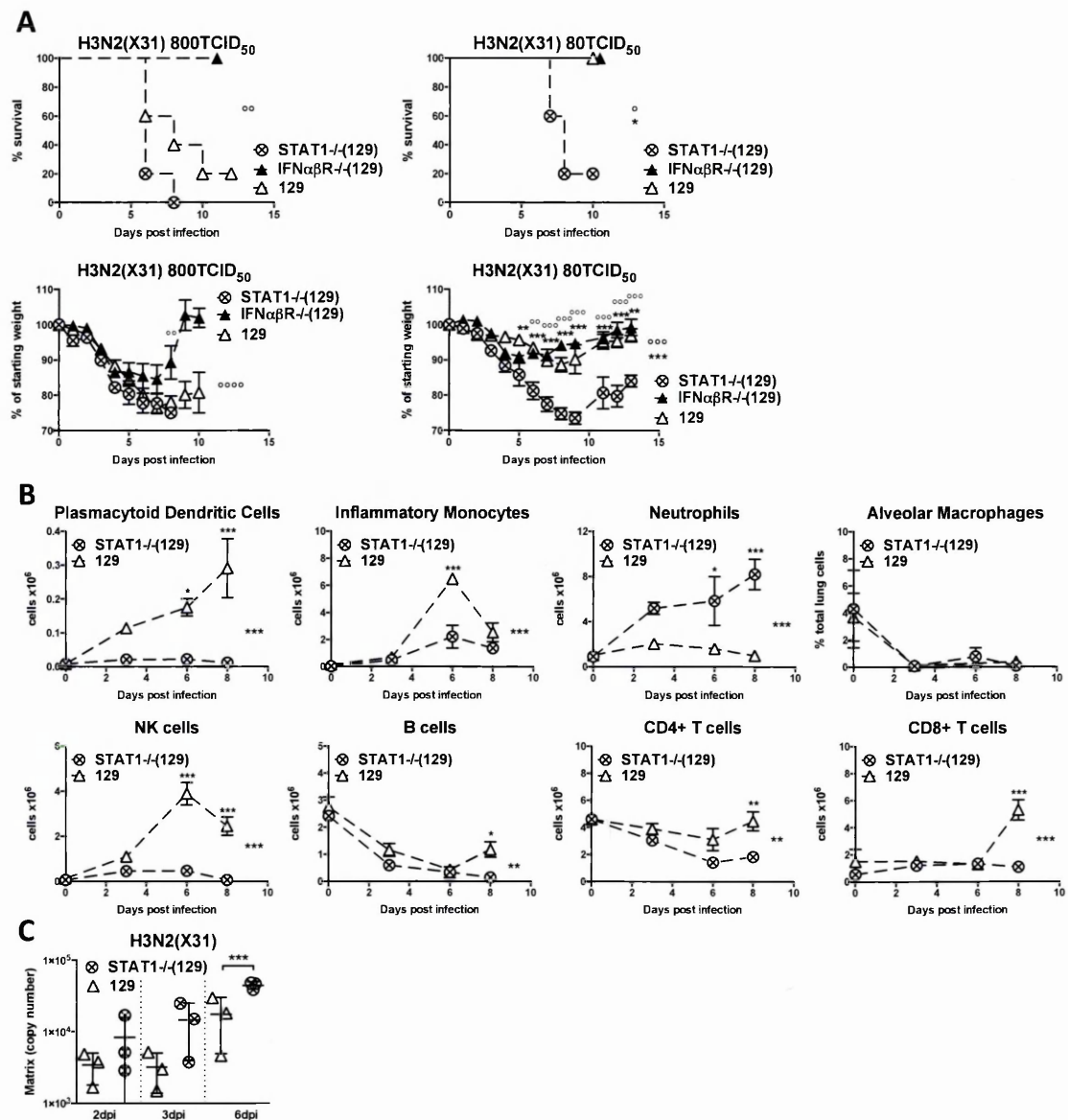


Figure 10: STAT1 is required for resistance to X31 induced disease. (A) STAT1^{-/-}(129, IFN α BR^{-/-}(129) and 129 mice were infected i.n. with 800TCID₅₀ (left panels) or 80 TCID₅₀ (right panels) of X31. Weight loss and survival were recorded throughout infection. (B-C) STAT1^{-/-}(129) and 129 mice were infected with X31: 80TCID₅₀ and flow cytometric quantification of pDCs, IMs, Neutrophils, AMs, NK cells, B cells, CD4⁺ T cells and CD8⁺ T cells was performed. (C) viral RNA present in infected lungs was quantitated by qPCR for the X31 Matrix gene on cDNA from whole lungs. Graphs show mean \pm s.e.m. and are representative of two independent experiments where n=3-6. *** indicates P<0.0001, ** P<0.001 and * P<0.01 by 2-way ANOVA (weight loss and immune cell recruitment) or Log-rank (Mantel-Cox) Test (survival). The symbols on the right of graphs indicate statistical significance of the whole curve, as tested by two-way ANOVA and symbols above specific points indicates significance between points as tested by Bonferroni post tests, where * denotes 129:STAT1^{-/-}(129) and ° IFN α BR^{-/-}(129):STAT1^{-/-}(129).

Figure 11

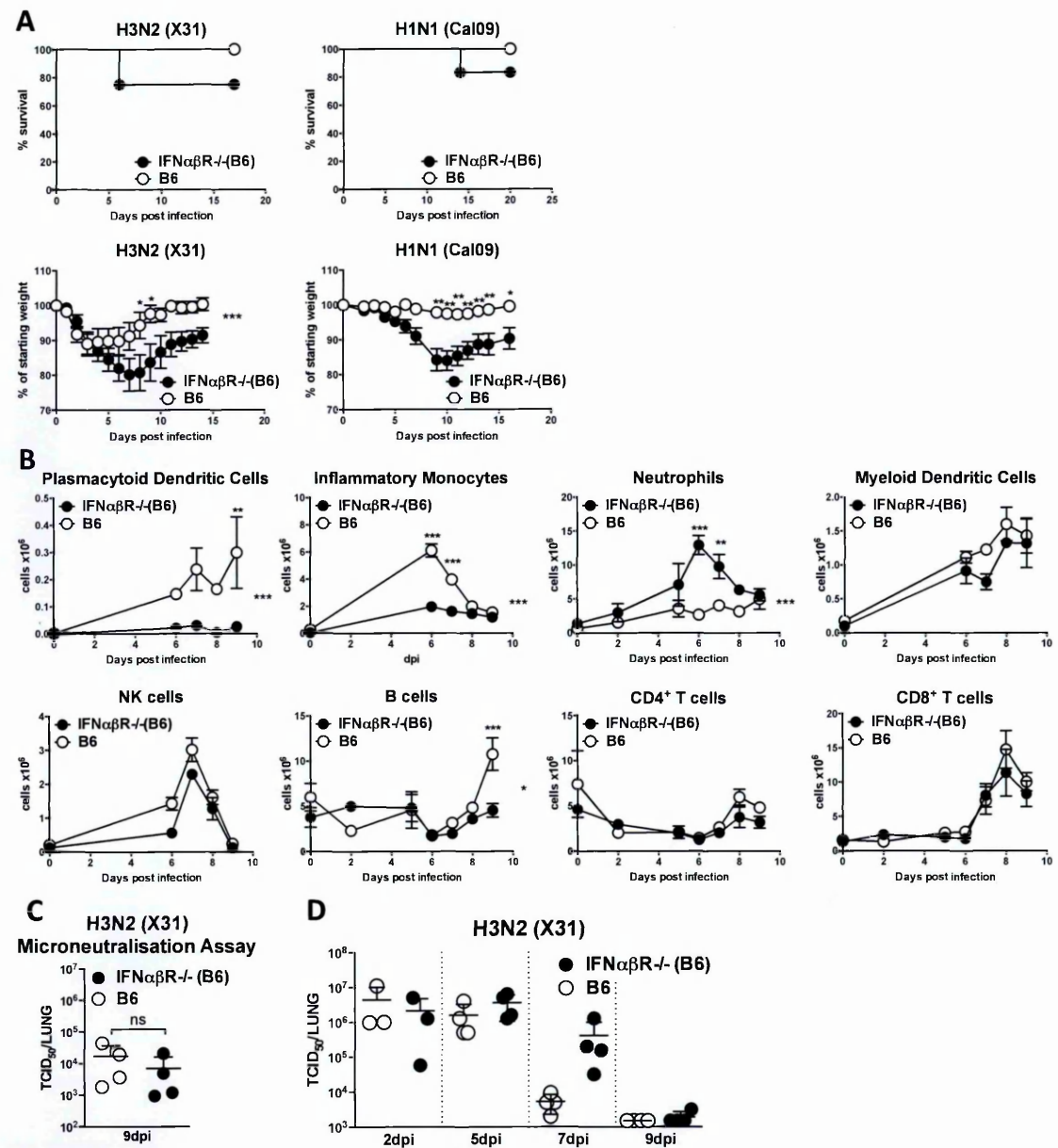


Figure 11: IFNαβR deficiency on a B6 background alters pulmonary inflammation and increases morbidity but not X31 induced mortality. (A) B6 (open circles) or IFNαβR-/- (B6) (black circles) mice were infected i.n. with X31: 8000 TCID₅₀ or with Cal09: 1000 TCID₅₀. Weight loss and mortality were measured. (B) B6 and IFNαβR-/- (B6) were infected with X31 and recruitment of pDCs, IMcs, Neutrophils, mDCs, NK cells, B cells, CD4⁺ T cells and CD8⁺ T cells was assessed by flow cytometry. (C) X31 neutralising antibody titre at 9dpi in serum was measured by microneutralisation assay. (D) Virus titers in lung homogenates taken at the indicated time points were measured by TCID₅₀ determination on MDCK cells. Graphs show mean ± s.e.m and are representative of 2 independent experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA (symbols to the right of graphs) with Bonferroni post tests (symbols above individual time points) (weight loss and cellular recruitment), Mann Whitney test (virus and antibody titre quantification), or Log-rank (Mantel-Cox) Test (survival).

CD4⁺ and CD8⁺ T cells and, in contrast to what was observed on the 129 background, NK cells was comparable between B6 and IFN α β R^{-/-}(B6) mice. B cell recruitment was depressed at later time points in IFN α β R^{-/-}(B6) mice in comparison to numbers observed in Wt lungs. However, lower numbers of B cells did not appear to impede induction of X31 specific antibodies, as sera tested for capacity to neutralise X31 from both genotypes at 9dpi was comparable (Figure 11B-C). X31 viral load in IFN α β R^{-/-}(B6) lung was higher at 7dpi than what was observed in B6 lungs, however this was not significant (Figure 11D). Perhaps as a result of persisting viral load in IFN α β R^{-/-}(B6) lungs, this genotype exhibited higher concentrations of proinflammatory cytokines, specifically: Eotaxin, G-CSF, IFN γ and IL-6 as well as elevated levels of IL-10, compared to what was observed in the Wt controls. There was no statistically significant difference between IL-9 concentrations in IFN α β R^{-/-}(B6) and B6 lungs. Interestingly, IP-10, MCP-1 and Mip-1 α secretion was significantly depressed at early time points of infection in IFN α β R^{-/-}(B6) mice, concentrations of these cytokines only reaching Wt levels late in infection (Figure 12). Thus, while IFN α β R deficiency on the B6 background blunts some aspects of innate immunity and this does correlate to increased morbidity of the host, ultimately this did not negatively impact on resolution of X31 infection or host survival.

In contrast, increasing type I IFN signalling during X31 infection in B6 mice by exogenous administration of recombinant IFN α 4 markedly increased host morbidity and mortality, while IFN α 4 treatment alone did not adversely affect B6 mice (Figure 13A). Increased host morbidity of X31 infected, IFN α 4 treated mice correlated with significantly higher numbers of IMCs and NK cells in the lung at

Figure 12

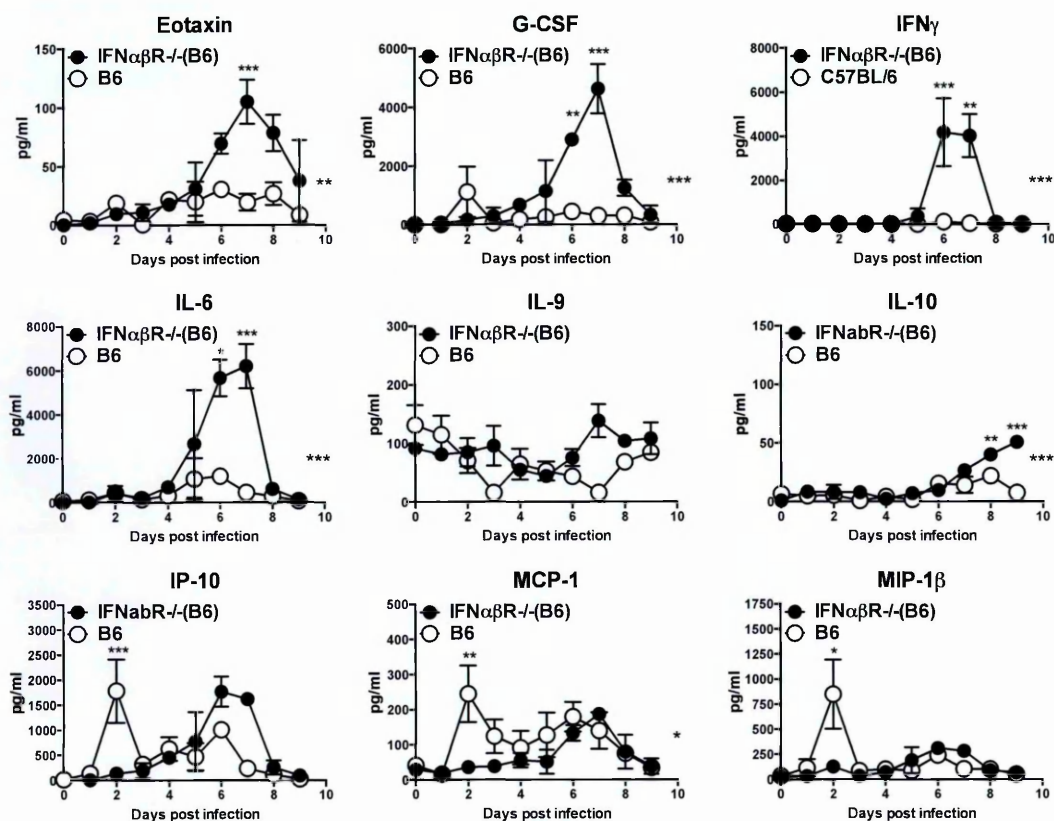


Figure 12: Comparison of X31 induced cytokine and chemokine secretion in B6 and IFN $\alpha\beta$ R $^{-/-}$ (B6) lungs. (A) B6 (open circles) or IFN $\alpha\beta$ R $^{-/-}$ (B6) (black circles) mice were infected i.n. with X31: 8000 TCID₅₀. Specified cytokine and chemokine concentrations were quantified by multiplex. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=3-4. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests. Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 13

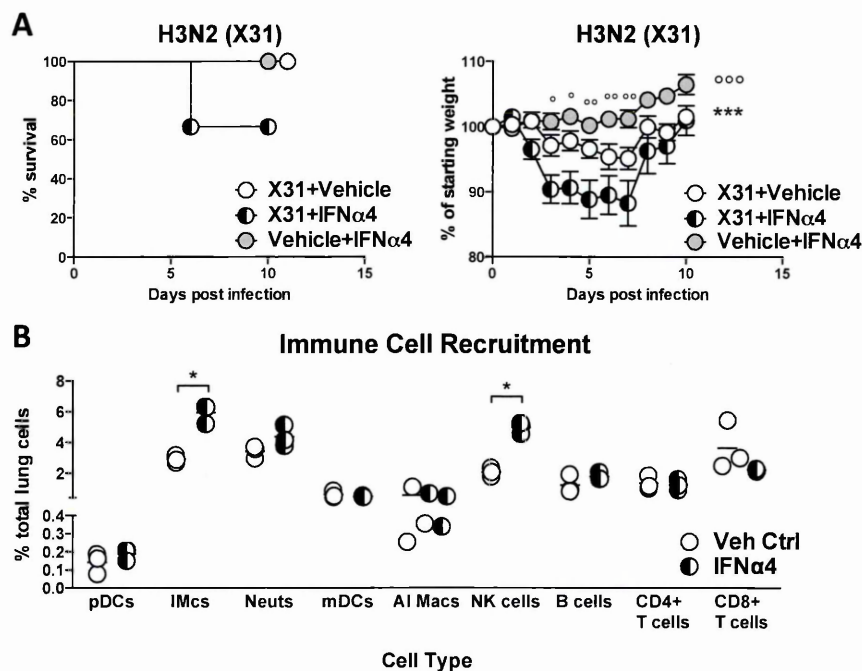


Figure 13: Exogenous administration of IFN α 4 increases susceptibility of low IFN α β responding mouse strain. B6 mice were infected i.n. with X31 (8000 TCID₅₀) (half filled and open circles) or mock infected (grey circles) and subsequently treated with mammalian IFN α 4 (3.5x10⁴U/200 μ L i.p) (half filled circles) or Veh Ctrl (open circles) every 24hrs from 1 to 6dpi. (A) Weight loss and mortality were recorded over time. (B) At 6dpi flow cytometric analysis of whole lungs was performed on X31 infected, Veh Ctrl treated and X31 infected, IFN α 4 treated groups. Recruitment of pDCs, IMcs, Neutrophils, mDCs, AMs, NK cells, B cells, CD4+ T cells and CD8+ T cells was assessed. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss) or Mann Whitney test (cellular recruitment) where * denotes X31+IFN α 4:X31+Veh Ctrl and ° denotes X31+IFN α 4:Veh Ctrl+IFN α 4. Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

6dpi, compared to infected mice treated with Veh Ctrl (Figure 13B). We also observed a slight increase in pDCs numbers recruited to the lungs of infected, IFN α 4 treated mice at this time point, however this was not statistically significant (Figure 13B). Collectively, these data demonstrate that while low-dose IFN $\alpha\beta$ responses are beneficial, high-dose IFN $\alpha\beta$ responses contribute to influenza-induced pathology.

3.3.5 Abundant hyper-reactive 129 pDCs produce excessive IFN $\alpha\beta$, but few pro-inflammatory cytokines

pDCs are potent type I IFNs producing cells and are recruited in high numbers to the lung during infection in 129 mice. Baseline analysis revealed that 129 mice have a higher frequency and number of pDCs in their lungs, spleen and mediastinal lymph node (LN) compared to B6 mice. Notably, genetic ablation of type I IFN signalling decreased pDC presence in all organs tested (Figure 14A).

We generated BM-derived Flt3L-pDCs from 129, B6 and IFN $\alpha\beta$ R^{-/-}(129) mice and stimulated these cells for 24hrs with X31 (MOI of 1). We assessed response to stimulus by measuring IFN α , β and λ secretion. Stimulation with live virus (X31) resulted in impressive amounts of IFN α , β and λ secreted from both 129 and B6 pDCs. Although response was robust from both strains, 129 pDCs consistently produced significantly more IFN α , β and λ than B6 pDCs (Figure 14B). These results confirm and extend similar observations made by Asselin-Paturel et al. (Asselin-Paturel et al., 2003).

Interestingly, stimulation of IFN $\alpha\beta$ R^{-/-}(129) pDCs with live X31 did not elicit secretion of IFN α , β or λ (Figure 13B). Furthermore, treatment of cells with an IFN $\alpha\beta$ R specific blocking antibody (MAR-1) resulted in no detection of IFN

Figure 14

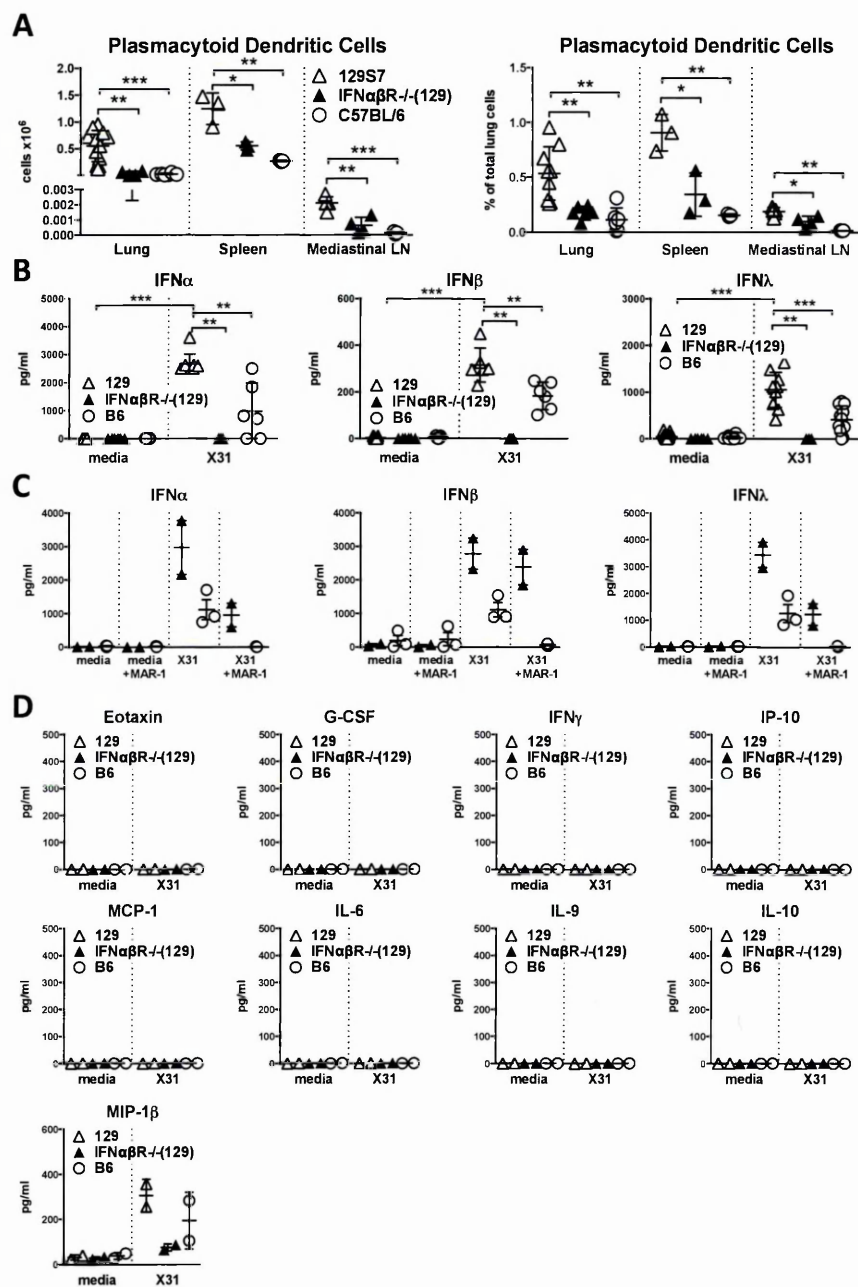


Figure 14: pDC frequency, number and capacity to secrete type I IFNs is elevated in the 129 strain and dependent upon IFN $\alpha\beta$ R. (A) pDC frequency and number in lung, spleen and mediastinal LN of 129 (open triangles), IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) and B6 (open circles) mice was assessed by flow cytometry. pDCs were defined as PDCA-1 $^{+}$, Siglec-H $^{+}$, CD11c $^{+}$, CD11b $^{-}$, FSC lo , SSC lo . (B) 129, IFN $\alpha\beta$ R $^{-/-}$ (129) and B6 BM-derived Flt3L driven pDCs from mice were stimulated in vitro for 24hrs with X31 (MOI of 1) and IFN α , β and λ secretion was measure by ELISA. (C) IFN α , β and λ response to X31 stimulation of Wt 129 and B6 pDCs the presence of MAR-1 (mAb for IFNAR1) was also assessed. (D) Secretion of stated cytokines by 129, B6 and IFN $\alpha\beta$ R $^{-/-}$ (129) pDCs was assessed by mulitplex. Graphs show mean \pm s.e.m and are pooled from 2-6 independent experiments, where n=2-4. Except for data in (C) which is representative of one experiment. *** indicates $p < 0.0001$, ** $p < 0.001$ and * $p < 0.01$ as assessed by Mann Whitney test.

secretion from X31 stimulated B6 pDCs. In contrast, MAR-1 treatment of X31 stimulated Wt 129 pDCs blunted, but did not entirely abolish IFN α and λ production and IFN β secretion appeared to be unaffected (Figure 14C). We also assessed whether or not pDCs make any other cytokines in response to IAV stimulation. We assayed for 32 common cytokines and chemokines, several of which were found in high concentrations in 129 lungs during infection, however with the exception of Mip-1 β , pDCs did not produce any cytokines in response to IAV stimulation. Interestingly, Mip-1 β secretion followed the same pattern as IFN production; highest from the 129 pDCs and lower in the B6, while IFN $\alpha\beta$ R $^{-/-}$ (129) pDCs did not respond (Figure 14D). These results suggest that there is a role for IFN $\alpha\beta$ R dependent feed-back in pDCs, not only IFN $\alpha\beta$ secretion but also in IFN λ and other cytokine production, furthermore as IFN $\alpha\beta$ R $^{-/-}$ pDCs did not secrete any cytokine in response to X31 stimulation, IFN $\alpha\beta$ R may be required for pDC maturation and/or function.

In light of increased responsiveness of pDCs in 129 mice and their presence in greater frequencies and numbers in 129 lungs at all time points examined after infection, we hypothesized that this cell type may be the prevailing source of IFN $\alpha\beta$ and therefore host pathology. Significantly, efficient depletion of pDCs in 129 mice using the mAb α PDCA-1 (Figure 15 and Figure 16F) markedly decreased host morbidity and mortality, which correlated with lower pulmonary concentrations of IFN α (Figure 16A-B), indicating that pDC-derived IFN α mediates host morbidity. IFN β levels were lower at 6dpi in α PDCA-1 treated lungs, however this was not statistically significant (Figure 16B). It is interesting to note that IFN λ levels are unchanged upon pDC depletion (Figure 16C), indicating other cell types for example, airway epithelial cells or other DCs, must contribute to the induction

Figure 15

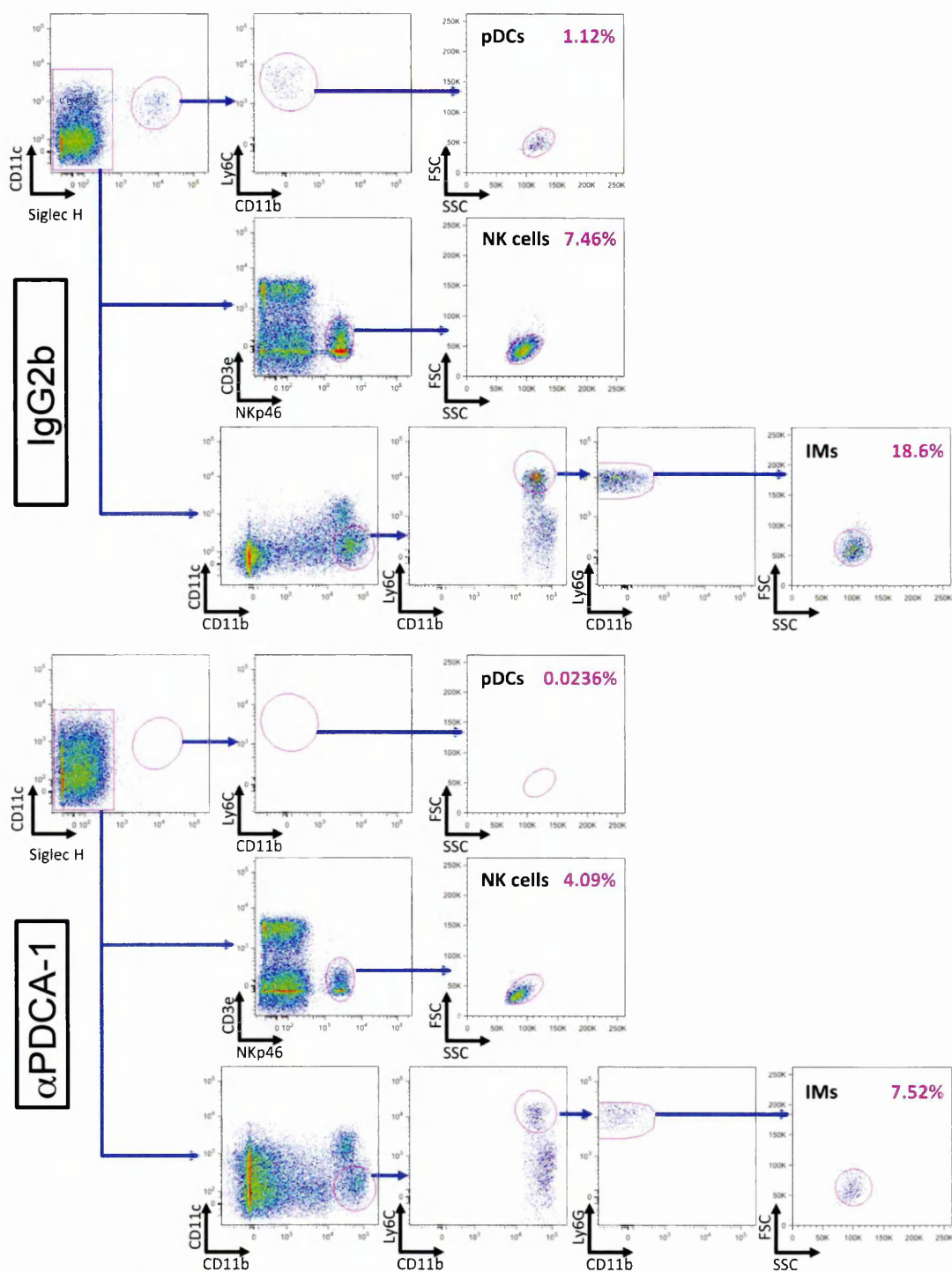


Figure 15: αPDCA-1 mAbs effectively depletes pDCs and lowers frequencies of other cell types present in the lung of infected 129 mice. 129 mice were treated with αPDCA-1 (500μg/200μL i.p) or isotype control (IgG2b) as indicated, and at 5dpi lung single cell suspensions were prepared. Gating strategies for flow cytometric quantification of pDCs (Siglec H⁺, CD11c⁺, Ly6C⁺, CD11b⁻, FSC^{lo}, SSC^{lo}), NK cells (NKp46⁺, CD3⁻, FSC^{lo}, SSC^{lo}) and inflammatory monocytes (Siglec H⁻, CD11b⁺, CD11c⁻, Ly6C^{hi}, Ly6G⁻, FSC^{int}, SSC^{int}) depicted using Flow Jo Version 9.5. Percentages indicate percent of live cells.

Figure 16

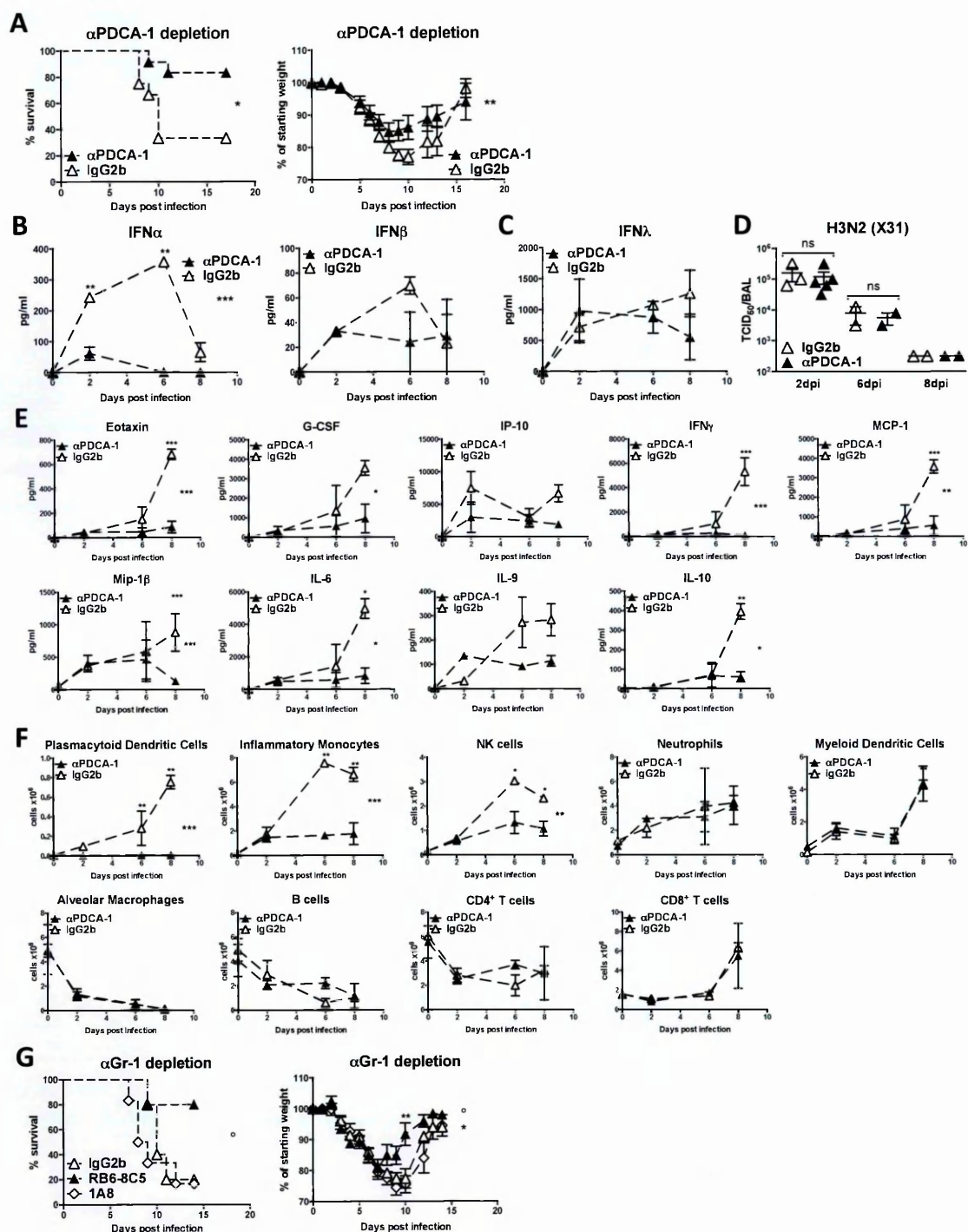


Figure 16: IFN $\alpha\beta$ derived from pDCs and other PDCA-1⁺ cells mediates inflammation and morbidity in infected 129 mice. (A-F) 129 mice were treated with depleting mAb α PDCA-1 (black triangles) or isotype control: IgG2b (open triangles) 24hrs prior to infection with X31: 800TCID₅₀ i.n, treatment was continued every 48hrs thereafter until at 10dpi. (A) Weight loss and mortality were measured throughout infection. (B-C) IFN α , β and λ protein in BAL fluid was quantified by ELISA, (D) viral titre in BAL fluid were determined, (E) presence of stated cytokines in BAL fluid was assessed by multiplex and

of this cytokine during infection. Moreover, high concentrations of IFN λ have been separated from high susceptibility to influenza-induced disease.

Depletion of PDCA-1⁺ cells and consequent reduction of IFN α in the pulmonary environment did not impede virus control. α PDCA-1 treated 129 mice cleared X31 at an equivalent rate to isotype controls, as assessed by titration of BAL fluid samples (Figure 16D). A reduction in IFN α early in X31 infection did however, correlate with decreased concentrations of proinflammatory cytokines in BAL fluid. Levels of Eotaxin, G-CSF, IP-10, IFN γ , MCP-1, Mip-1 β , IL-6 IL-10 and IL-9 were all reduced in α PDCA-1 treated 129 mice to concentrations reminiscent of what was observed in IFN $\alpha\beta$ R^{-/-}(129) mice (Figure 16E). Moreover, α PDCA-1 treatment significantly decreased the frequencies (not shown) and numbers of IMcs and NK cells recruited to the infected lungs. However, treatment did not affect Neutrophil, mDC, Alveolar Macrophage, B cell, CD4⁺ T cell and CD8⁺ T cell numbers in the pulmonary environment at any time point assessed during X31 infection (Figure 16F).

Aside from constitutive expression on pDCs, PDCA-1 has also been shown to be upregulated on certain cell types upon IFN $\alpha\beta$ stimulation, thus this decrease in

Figure 16 (cont): (F) cell recruitment of pDCs, IMcs, NK cells, Neutrophils, mDCs, AMs, B cells, CD4⁺ T cells and CD8⁺ T cells was assessed by flow cytometry. (G) 129 mice were treated with depleting mAbs RB6-8C5 (filled triangles), 1A8 (open diamonds) or isotype control (open triangles) then infected with X31, treatment was continued every 48hrs thereafter, weight loss and survival was recorded. Graphs show mean \pm s.e.m and are representative of 2-3 independent experiments where n=2-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss, cytokine concentrations and cellular recruitment), Mann Whitney test (viral titre quantification), or Log-rank (Mantel-Cox) Test (survival) where * denotes α PDCA-1:IgG2b or RB6-8C5:IgG2b and ° denotes RB6-8C5:1A8. Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

numbers of IMcs and NK cells may not just be due to reduced cytokine levels but also due to direct depletion of monocytes and NK cells that upregulate PDCA-1 upon stimulation with type I IFNs. We also employed the mAb: RB6-8C5, which is specific for the antigen Gr-1 that is composed of Ly6C (expressed on pDCs and IMcs) and of Ly6G (expressed on neutrophils). Treatment of 129 mice with this mAb-depleted pDCs, IMcs and Neutrophils and resulted in a drastic reduction in IAV-induced morbidity and mortality. Improved 129 resistance was not due to neutrophil depletion, as the neutrophil-specific mAb 1A8 did not change the course of disease (Figure 16G).

The decrease in inflammatory cell recruitment and proinflammatory cytokine secretion is highly reminiscent of cytokine and chemokine levels and cellular recruitment found in $\text{IFN}\alpha\beta\text{R}^{-/-}$ (129) mice during X31 infection. Collectively, these data indicates that 129 mice experience a more vigorous type I IFN response through the combined effect of more pDCs at base line, higher recruitment into the lung on infection and higher responsiveness on a per-cell basis of recruited pDCs and, potentially, other PDCA-1+ cells. We place pDC-derived $\text{IFN}\alpha\beta$ upstream of the intense inflammatory response observed in the 129 strain during IAV infection.

3.3.6 Strain-dependent differences in susceptibility are mediated by innate immunity

129 mice demonstrate higher susceptibility to influenza-induced disease, yet they are able to control X31 replication (Figure 8A) and recruitment of both CD4^{+} and CD8^{+} T cells to 129 lungs is comparable to the recruitment of these cell types observed in resistant B6 and $\text{IFN}\alpha\beta\text{R}^{-/-}$ (129) strains (Figure 7E). Although recruitment of influenza specific CD8^{+} T cells was comparable between strains,

higher numbers of activated (CD69⁺) CD8⁺ T cells were observed in 129 lungs (Figure 17A). An over activation of the cytotoxic T cell response may explain increased 129 strain susceptibility. To ascertain whether the adaptive immune response contributes to differences in influenza resistance we compared Rag-deficient 129 and B6 mice, which are deficient for B and T cells. X31 infected Rag^{-/-}(129) mice, like their Wt counterparts, lost weight more rapidly and reached clinical end point before 10dpi. In contrast, weight loss in Rag^{-/-}(B6) mice progressed at a significantly slower rate, plateaued from 6 to 10dpi before plunging again until 14-15dpi where mice reached clinical end point (Figure 17B). Interestingly, virus quantification at 9dpi, when Rag^{-/-}(129) mice are at clinical endpoint yet all Rag^{-/-}(B6) mice are still alive, showed no differences in X31 matrix copy number in the lung (Figure 17C). 129 susceptibility to IAV induced disease therefore appears independent of an over activated adaptive immune response and high virus load. Rag^{-/-}(129) morbidity did however correlate with higher concentrations of IFN α , β and λ in the BAL fluid (Figure 17D). Lastly, specific depletion of CD8⁺ T cells using an α CD8 mAb did not alter 129 disease course (Figure 17E). Thus, while type I IFN signalling leads to higher numbers of activated CD8⁺ T cells in 129 lung during X31 infection this phenomenon does not appear to significantly contribute to 129 IAV-induced pathology.

Unlike T cell recruitment, B cell numbers in 129 lungs during IAV infection were markedly lower than what was recorded in the resistant B6 and IFN $\alpha\beta$ R^{-/-}(129) strains (Figure 7C). In line with lower B cell recruitment, 129 sera had less influenza specific antibody titres than titres found in IFN $\alpha\beta$ R^{-/-}(129) and B6 sera (Figure 7F), however this did not lead to increase sensitivity of 129 mice to homologous IAV challenge. 129, IFN $\alpha\beta$ R^{-/-}(129) and B6 mice were all given a mild

Figure 17

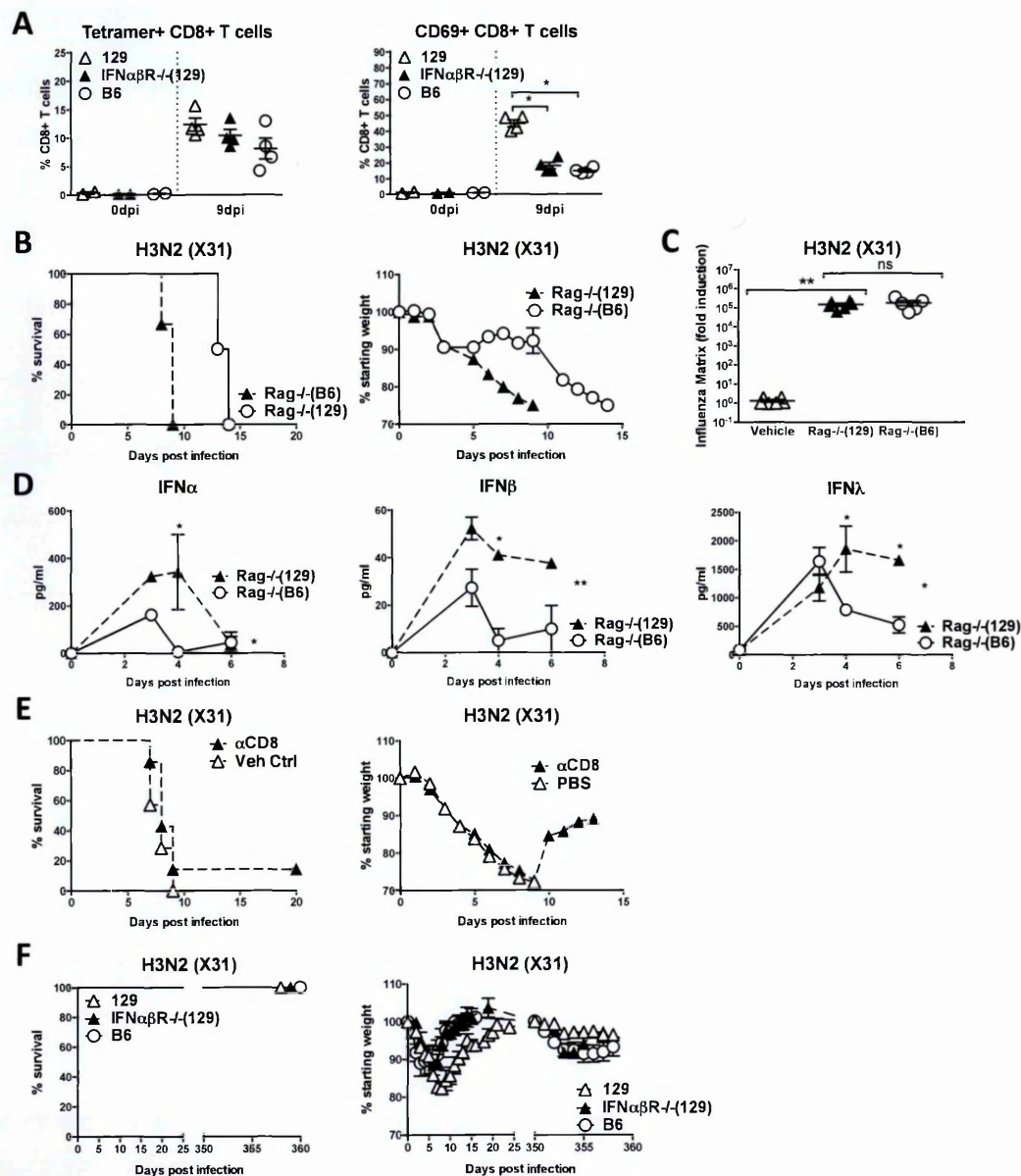


Figure 17: Increased IAV susceptibility of 129 mice is independent of adaptive immunity. (A) 129 (open triangles), IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) and B6 (open circles) mice were infected with X31: 800TCID $_{50}$ i.n. and at 8dpi lung single cell suspensions were prepared and flow cytometric quantification of influenza specific (Tetramer+) and activated (CD69+) CD8+ T cells was performed. (B-D) Rag $^{-/-}$ (129) (black triangles) and Rag $^{-/-}$ (B6) (open circles) mice were infected with X31 (800TCID $_{50}$), (B) weight loss and mortality recorded, (C) virus present in the lung at 9dpi was determined by qPCR on total lung RNA and (D) BAL levels of IFN α , β and λ were assessed by ELISA. (E) 129 mice were treated with the mAb α CD8 (black triangles) or isotype control (open triangles) 24hrs prior to infection and again at 4dpi, mice were infected with X31: 800TCID $_{50}$ and weight loss and survival was assessed. (F) 129 (open triangles), IFN $\alpha\beta$ R $^{-/-}$ (129) (black triangles) and B6 (open circles) mice were infected with X31: 800TCID $_{50}$ i.n. and at 350dpi mice were challenged with X31: 1.5x10 6 TCID $_{50}$ i.n., survival and morbidity were recorded. Graphs show mean \pm s.e.m and are representative of 2 independent

dose of X31: 80TICD₅₀ and left to recover, at 350dpi mice were challenged with X31: 1.5x10⁶TCID₅₀ and all mice survived, exhibiting very low morbidity. Surprisingly, IFN α β R^{-/-}(129) and B6 mice lost more weight than the 129 strain however this may be indicative of the comparatively lower severity of primary infection (Figure 17F).

To further confirm that exaggerated IFN α β signalling was not driving 129 pathology through effects on the adaptive immune system we generated mixed bone marrow chimeras. Host Rag^{-/-}(129) mice were irradiated and their hematopoietic cells reconstituted with Rag^{-/-}(129) bone marrow supplemented with 10% of cells from either Wt 129 or IFN α β R^{-/-}(129) bone marrow. We performed flow cytometric assessment of the adaptive immune compartment eight weeks after reconstitution and observed comparable reconstitution of CD4⁺ and CD8⁺ T cells and mature B cells (B220⁺, CD19⁺, IgM⁺, IgD⁺, FSC^{lo}, SSC^{lo}) in the lung and spleen, as well as equivalent frequencies of immature B cells (B220⁺, CD19⁺, IgM⁺, IgD⁻, FSC^{lo}, SSC^{lo}), pre B cells (B220⁺, CD19⁺, IgM⁻, FSC^{lo}, SSC^{lo}), Follicular B cells (B220⁺, CD93⁻, CD23⁺, IgM⁺, FSC^{lo}, SSC^{lo}), Marginal Zone B cells (B220⁺, CD93⁻, CD23⁻, IgM⁺, FSC^{lo}, SSC^{lo}) and Germinal Centre B cells (B220⁺, PNA⁺, GL7⁺, FSC^{lo}, SSC^{lo}) in the spleen in both sets of chimeras (Figure 18A). Upon X31 infection CD4⁺ T cells, CD8⁺ T cells and mature B cells were recruited to the lung in comparable numbers, irrespective of presence of IFN α β R on these cell types (Figure 18B).

Figure 17 (cont): experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss and cytokine concentrations), Mann Whitney test (viral titre quantification), or Log-rank (Mantel-Cox) Test (survival). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 18

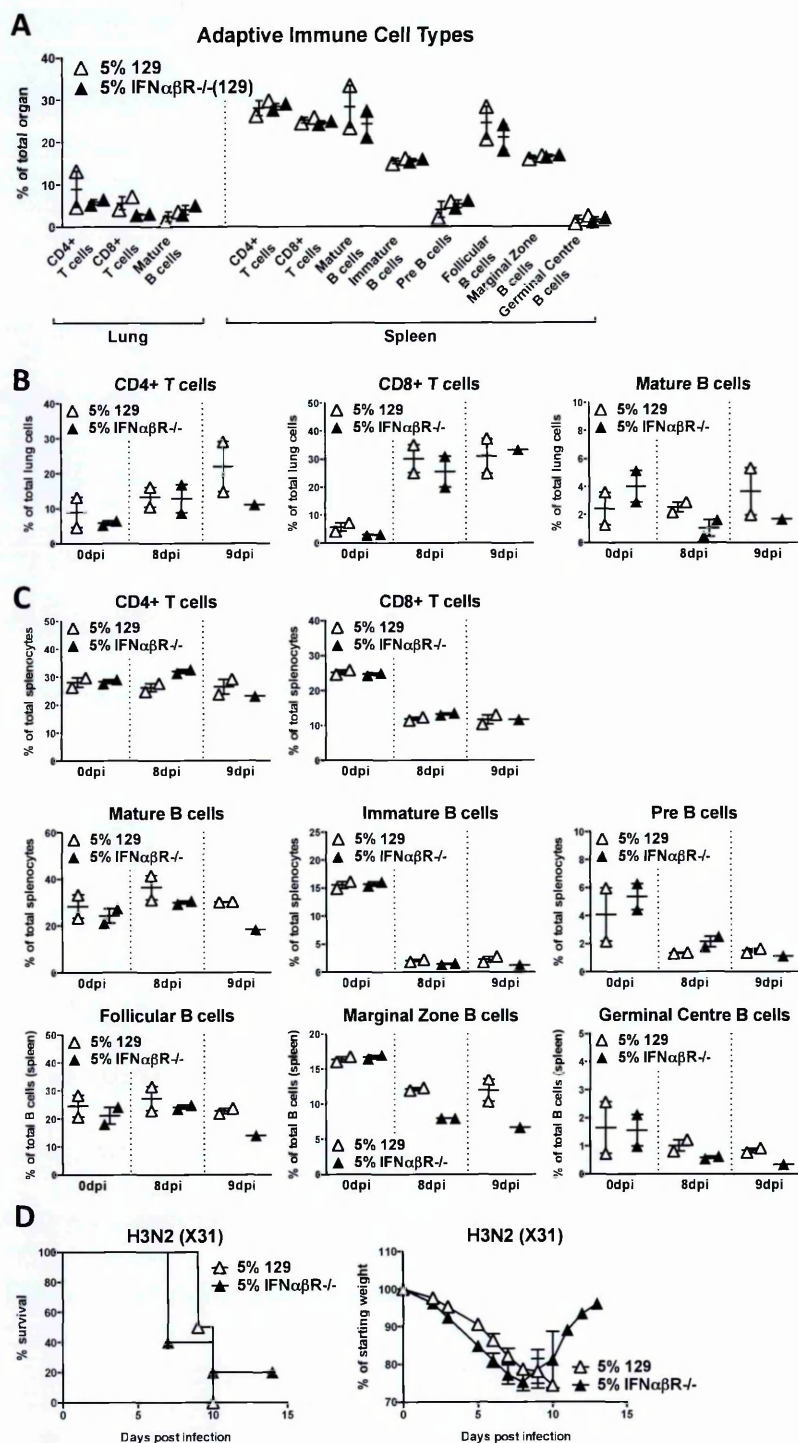


Figure 18: IFN $\alpha\beta$ R deficient adaptive immune cells do not ameliorate X31 induced disease in 129 mice. (A-D) Host Rag $^{-/-}$ (129) mice were irradiated and their hematopoietic cells reconstituted with Rag $^{-/-}$ (129) bone marrow supplemented with 5% of cells from 129 (5% 129, open triangles) or IFN $\alpha\beta$ R $^{-/-}$ (129) (5% IFN $\alpha\beta$ R $^{-/-}$ (129), black triangles) bone marrow. (A) Reconstitution of adaptive immune cell types was assessed at 6 weeks post irradiation, flow cytometry was used to determine frequency of CD4 $^{+}$ T cells, CD8 $^{+}$ T cells and Mature B cells in lungs and spleen as well as Follicular B cells, Marginal

Furthermore, while frequencies of all adaptive immune cell types tested changed in the spleen due to infection, this was not affected by the presence of IFN $\alpha\beta$ R (Figure 18C). Overall, an IFN $\alpha\beta$ R $^{-/-}$ adaptive immune compartment did not ameliorate IAV-induced pathology in 129 mice as infection with X31 resulted in high morbidity and mortality of both chimera groups (Figure 18D).

Although we were confident that 129 susceptibility to IAV is not intimately linked to IFN $\alpha\beta$ effects on the adaptive immune system, we were interested in the reduced B cell response observed in Wt 129 mice. We wondered if IFN $\alpha\beta$ R deficiency could improve B cell function or survival in a high IFN $\alpha\beta$ environment. To assess whether or not IFN $\alpha\beta$ R $^{-/-}$ (129) B cells had a competitive advantage over Wt 129 B cells we created mixed chimeras in Rag $^{-/-}$ (129) hosts reconstituted with 90% Rag $^{-/-}$ bone marrow plus 5% CD45.1 $^{+}$ 129 bone marrow and 5% CD45.2 $^{+}$ IFN $\alpha\beta$ R $^{-/-}$ (129) bone marrow. Using the congenic markers: CD45.1 and CD45.2, we were able to trace the origin of adaptive immune cells. Analysis by flow cytometry of the lung and spleen samples after reconstitution showed that CD45.1 $^{+}$ (Wt) cells had a slight competitive advantage over CD45.2 $^{+}$ IFN $\alpha\beta$ R $^{-/-}$ (129) cell types in reconstitution of the adaptive immune cell compartment. CD4 $^{+}$ and CD8 $^{+}$ T cells and mature B cells in the lung and CD4 $^{+}$ and CD8 $^{+}$ T cells and mature, immature, pre, follicular, marginal zone and germinal centre B cells in the spleen,

Figure 18 (cont): Zone B cells and Germinal Centre B cells in the spleen. (B-D) Bone marrow chimeras were infected with X31 (80TCID₅₀) and (B-C) frequencies of stated cell types was assessed by flow cytometry in the (B) lung and (C) spleen. (D) Weight loss and mortality was measured throughout infection. Graphs are representative of 1 experiment where n=2-6. Statistical significance was assessed by 2-way ANOVA with Bonferroni post tests (weight loss), Mann Whitney test (immune cell frequency), or Log-rank (Mantel-Cox) Test (survival). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 19

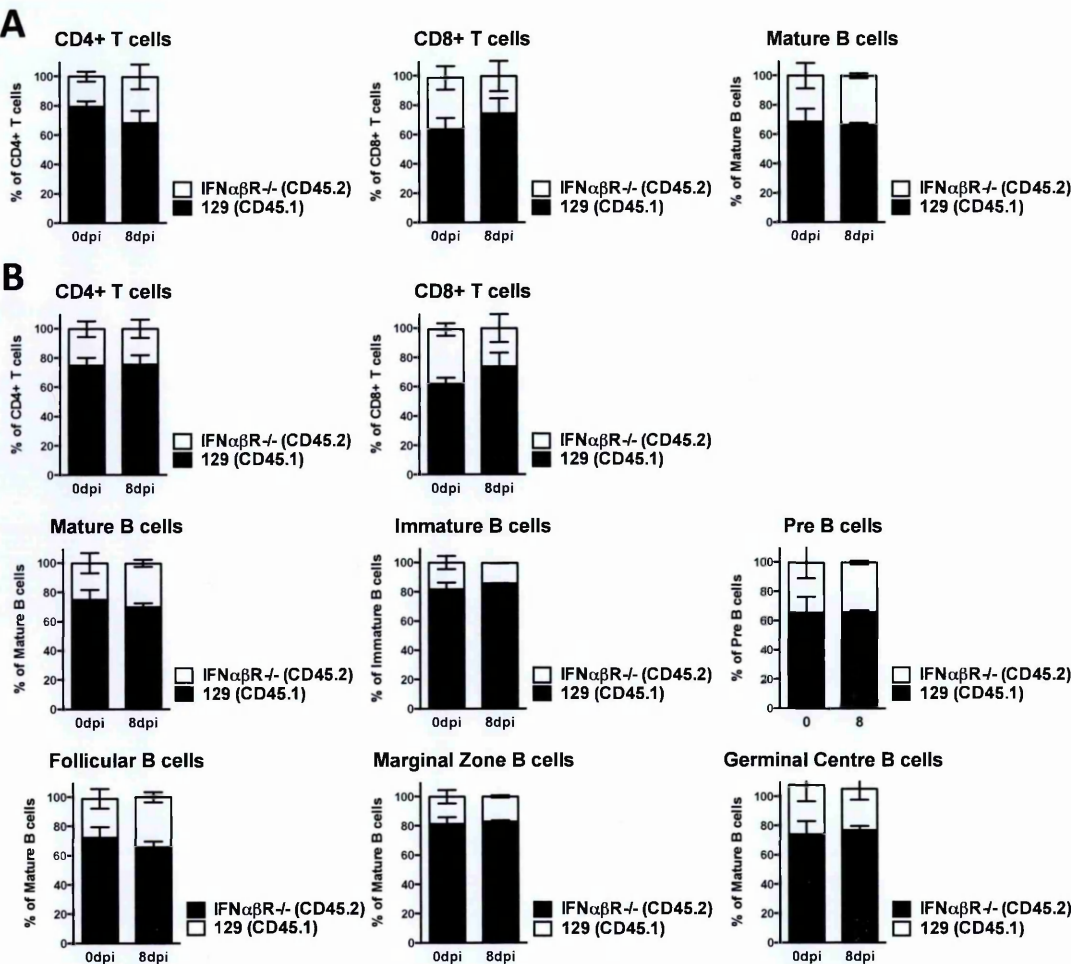


Figure 19: IFNαβR deficiency on cells of the adaptive immune compartment does not confer a competitive advantage for reconstitution or during X31 infection. (A-B) Rag-/- (129) mice were reconstituted with 90% Rag-/- (129) bone marrow, 5% CD45.1+ 129 bone marrow and 5% CD45.2+ IFNαβR-/- (129) bone marrow. 8 weeks after chimera generation mice were infected with X31: 80TCID₅₀ and samples were taken at 0 and 8 dpi. Flow cytometry was used to assess proportion of CD45.1+ (Wt) (open bar) and CD45.2+ (IFNαβR-/- (129)) (black bar) cells within (A) CD4+ T cell, CD8+ T cell and mature B cell populations in the lung and (B) CD4+ T cell, CD8+ T cell, mature B cell, immature B cell, Pre B cell, Follicular B cell, Marginal Zone B cell and Germinal Centre B cell populations in the spleen. Graphs are representative of 1 experiment where n=2. Statistical significance was assessed by Mann Whitney test.

all trended to be of Wt origin, approximately 60-80% of all these cell types being CD45.1⁺. Furthermore, X31 infection did not appear to alter this ratio, as proportions of all tested cell types in both the lung and spleen did not change when assessed at 8dpi (Figure 19). Taken together these results strongly indicates that differences in 129, IFN α β R^{-/-}(129) and B6 susceptibility to influenza induced disease is not due to differences in their respective adaptive immune responses and does not correspond to viral control.

3.3.7 NK cell depletion does not protect 129 mice from severe IAV-induced disease

NK cell recruitment is augmented in 129 lungs during X31 infection. NK cells can become over activated and cause tissue damage in some settings (Okamoto et al., 2002). We therefore decided to test whether NK cells contribute downstream to 129 pathology. Using AsilaoGM, we efficiently depleted NK cells from the lung prior to infection of 129 mice with X31 (Figure 20A). NK cell depletion did not alter disease outcome, as AsilaoGM treated 129 mice succumbed to IAV-induced disease at a similar rate to the Vehicle Control (Veh Ctrl) group (Figure 20B).

3.3.8 Type I IFN mediated upregulation of TRAIL and DR5 induces epithelial cell death and therefore host susceptibility

TNF related apoptosis-inducing ligand (TRAIL) has been demonstrated to induce apoptosis of cells through the interaction with its receptor death receptor 5 (DR5) (Schaefer et al., 2007). Furthermore, TRAIL has been shown to be upregulated by many cell types in response to type I IFN stimulation (Hogner et al., 2013; Santini et al., 2000). We therefore hypothesised that immune cell expression

Figure 20

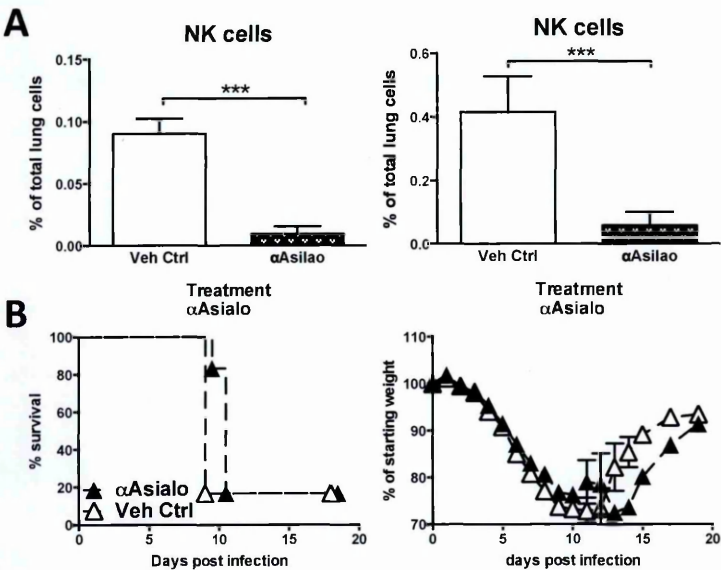


Figure 20: Depletion of NK cells does not protect 129 mice from X31 induced pathology. 129 mice were treated with AsilaoGM or Veh Ctrl 24hrs prior to X31 (800TCID₅₀) infection and again at 4dpi. (A) Flow cytometric analysis of NK cell depletion from the lung was performed prior to infection, NK cells were defined as NKp46⁺, CD3⁻, FSC^{lo}, SSC^{lo}. (B) X31 induced weight loss and survival was recorded. Graphs are representative of 2 independent experiment where n=2-6. Statistical significance was assessed by Mann Whitney test (NK cell frequency and number), 2-way ANOVA with Bonferroni post tests (weight loss), or Log-rank (Mantel-Cox) Test (survival).

of TRAIL may be mediating lung tissue damage. We assessed TRAIL expression on a range of cell types recruited to the lung during IAV infection and found that TRAIL was not expressed by NK cells, CD8⁺ T cells, CD4⁺ T cells, B cells or pDCs at the time points assessed (data not shown). However, TRAIL expression on IMCs was highly up regulated in 129 mice and this is dependent upon IFN $\alpha\beta$ signalling. Genetic ablation of IFN $\alpha\beta$ signalling abolished this upregulation of TRAIL on IMCs (Figure 21A).

129 epithelial cells (defined as Ecadherin⁺, CD45⁻) increased their surface expression of the TRAIL receptor DR5, whereas epithelia insensitive to IFN $\alpha\beta$ signalling did not (Figure 21B). Concurrently, we observed a higher frequency of cell death in 129 epithelia, as compared to IFN $\alpha\beta$ R^{-/-}(129) epithelia (Figure 21C). TUNEL staining on histological sections from infected 129 and IFN $\alpha\beta$ R^{-/-} lungs at 7dpi also confirmed a higher incidence of apoptotic epithelial cells in Wt 129 mice compared to IFN $\alpha\beta$ R^{-/-}(129) mice (Figure 21D).

To examine whether or not TRAIL/DR5 interaction contributes to epithelial cell death and morbidity of 129 mice, we treated X31 infected 129 mice with a blocking mAb for TRAIL (α TRAIL). Blockade of TRAIL interaction with DR5 throughout IAV infection resulted in reduced weight loss and mortality (Figure 22A). Consistent with this increased resistance to influenza-induced disease, α TRAIL-treated 129 mice had a significantly lower frequency of airway epithelial cell death at 7dpi. However, α TRAIL treatment did not alter DR5 expression on epithelial cells (Figure 22B). These results indicate that during IAV infection, IFN $\alpha\beta$ induces expression of TRAIL on monocytes recruited into the lung and of DR5 on lung epithelia, and that the interaction of these molecules leads to

Figure 21

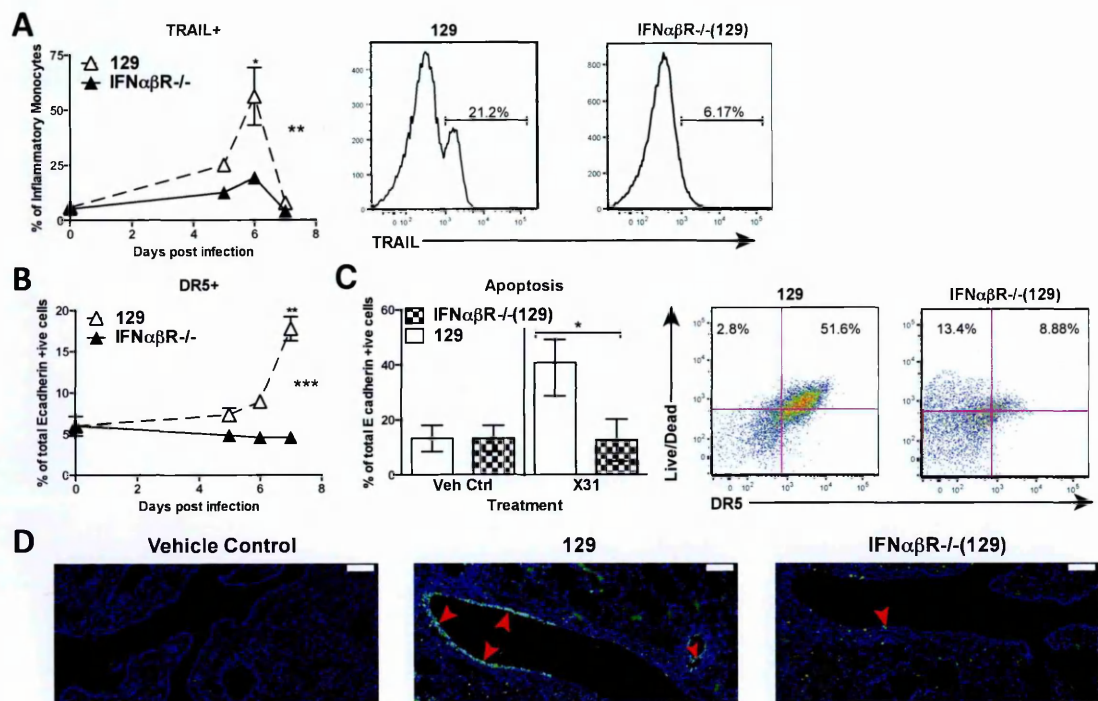


Figure 21: IFNαβ is upstream of TRAIL:DR5-expression in X31 infected 129 mice. 129 (open triangles) and IFNαβR-/- (129) (black triangles) mice were infected with X31 (800TCID₅₀). Flow cytometric analysis of expression levels of (A) TRAIL on pulmonary IMCs, (histograms show expression at 6dpi) and (B) DR5 on airway epithelial cells throughout X31 infection. (C) At 7dpi epithelial cells were assessed for free amine staining as a measure of cell death. Dot plots show the correlation between free amine and DR5 expression. (D) Lung sections from control and infected mice of the indicated genotypes were stained by TUNEL for apoptotic cells. Red arrowheads indicate TUNEL signal. Scale bar, 100 μm. Graphs show mean ± s.e.m and are representative of 2 independent experiments where n=3-4. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (TRAIL or DR5 expression) or by Mann Whitney test (frequency of apoptotic epithelial cells). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 22

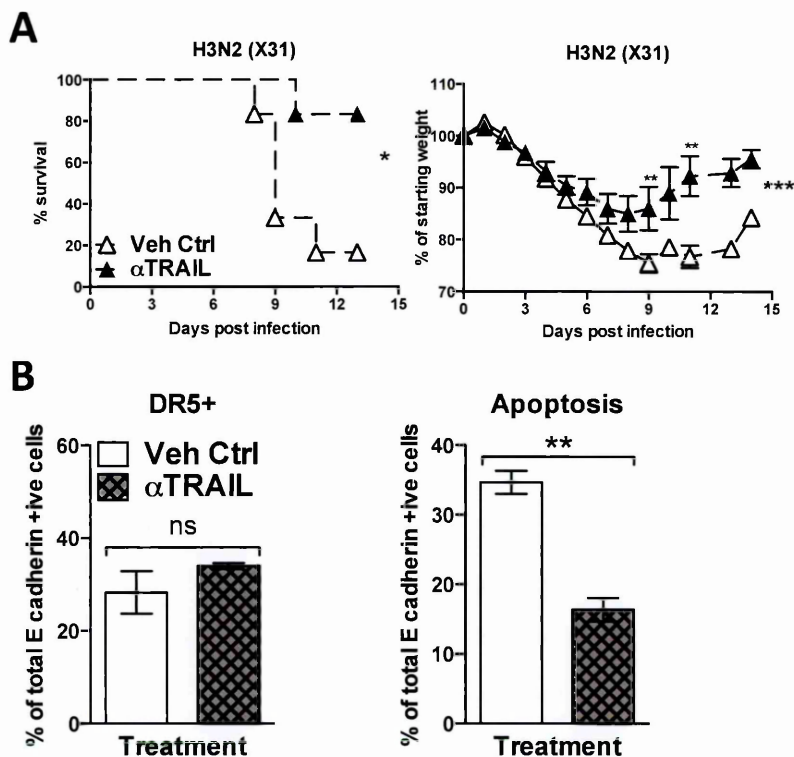


Figure 22: Blockade of TRAIL-DR5 interaction protects 129 mice from X31 induced disease. 129 mice were treated with α TRAIL (150 μ g/200 μ l i.p.) (black triangles) or Veh Ctrl (open triangles) at 1 day prior to infection with X31: (800TCID₅₀) and every 24hrs thereafter until 10dpi. (A) Mortality and morbidity was recorded throughout infection. (B) At 7dpi DR5 expression on airway epithelial cells and frequency of epithelial cell death (cells positive for free amines) was measured by flow cytometry. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss), by Mann Whitney test (frequency of apoptotic epithelial cells) or Log-rank (Mantel-Cox) Test (survival). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

epithelial cell death. Blockade of this interaction protects from the severe disease observed in IAV-infected 129 mice.

To confirm that TRAIL and DR5 expression is dependent on IFN $\alpha\beta$ R and not a result of severe disease, we also assessed expressions of TRAIL on IMCs and DR5 on epithelial cells in STAT1 $^{-/-}$ (129) mice. IFN $\alpha\beta$ is unable to signal without STAT1 and as shown in Figure 10 STAT1 $^{-/-}$ (129) mice are exquisitely susceptible to IAV-induced disease. Both TRAIL expression on IMCs and DR5 expression on epithelial cells were not upregulated during X31 infection, as assessed by flow cytometry (Figure 23A). Furthermore, the low IFN-responding, IAV-resistant mouse strains; B6 and BALB/C did upregulate TRAIL on IMCs and DR5 on epithelia, albeit not to the extent of the 129 strain. The comparatively lower TRAIL and DR5 expression in the resistant strains correlated to a lower frequency of epithelial cell death (Figure 23B). IFN $\alpha\beta$ R $^{-/-}$ (B6) mice, which are slightly more susceptible to IAV-induced disease than the B6 parental strain, did not exhibit any upregulation of TRAIL on IMCs or DR5 on epithelial cells during X31 infection. Interestingly, in spite of a comparatively higher disease burden, frequency of epithelial cell death at days 6 and 7 post infection was lower in IFN $\alpha\beta$ R $^{-/-}$ (B6) lungs compared to Wt controls (Figure 23C). Finally, treatment of B6 mice with IFN α 4 during X31 infection increased TRAIL expression on IMCs and DR5 expression on epithelial cells and this associated with increased frequency of airway epithelial cell death (Figure 23D) and host morbidity (Figure 13A). Collectively, these results demonstrate that TRAIL and DR5 expression is IFN $\alpha\beta$ dependent and not a function of severe disease burden.

We hypothesised that type I IFN induced expression of DR5 on epithelial cells allowed for substantial cell death in the lung epithelia layer and leads to host

Figure 23

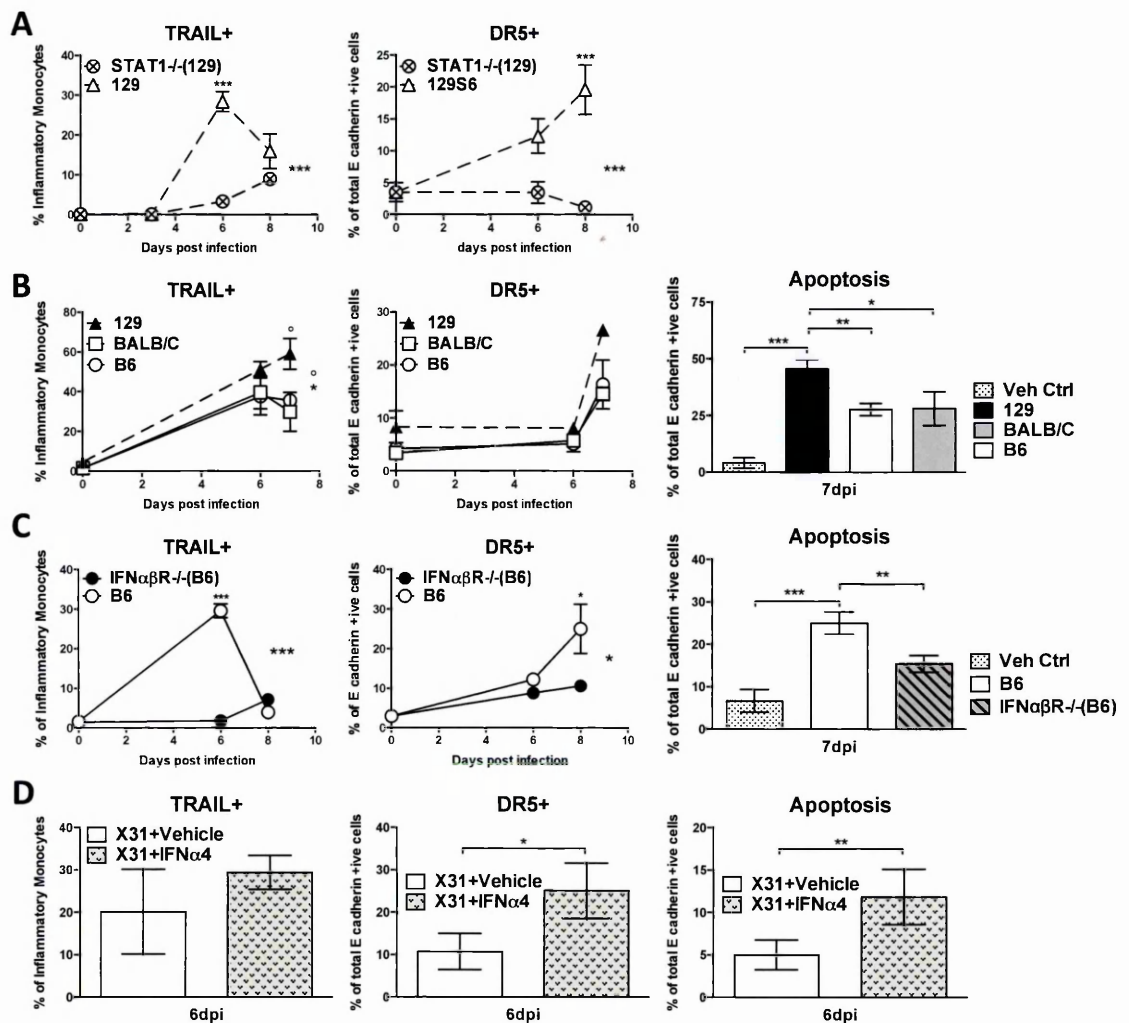


Figure 23: Upregulation of TRAIL and DR5 is dependent upon IFN $\alpha\beta$ signalling, not disease burden. (A-D) Single cell suspensions were extracted from whole lungs from specified mice at specified time points and flow cytometric analysis of TRAIL expression on IMCs, DR5 expression on epithelial cells and quantification of apoptotic epithelial cells was performed. (A) 129 (open triangles) and STAT1 $^{-/-}$ (129) (crossed circles) mice were infected with X31 (800TCID $_{50}$). (B) 129 (black triangles), B6 (open circles) and BALB/C (open squares) were infected with X31: 8000TCID $_{50}$. (C) B6 and IFN $\alpha\beta$ R $^{-/-}$ (B6) (black circles) mice were infected with X31: 8000TCID $_{50}$. (D) B6 mice were infected with X31: 8000TCID $_{50}$ and treated with exogenous IFN α 4 or Veh Ctrl from day 1-5 post infection, at 6dpi lungs were assessed as stated above. Graphs show mean \pm s.e.m and are representative of 2 independent experiments, where n=3-4, with the exception of (D), which is representative of one experiment, where n=3. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (TRAIL or DR5 expression) or by Mann Whitney test (frequency of apoptotic epithelial cells and (D)). For (B) * indicates 129:B6 and \circ denotes 129:BALB/C. Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

pathology. To test this, we generated bone marrow chimeras using IFN $\alpha\beta$ R deficient mice as hosts, re-populated by IFN $\alpha\beta$ R^{+/+} bone marrow cells (129>IFN $\alpha\beta$ R^{-/-}(129)) and vice versa (IFN $\alpha\beta$ R^{-/-}(129)>129), plus appropriate controls (129>129 and IFN $\alpha\beta$ R^{-/-}(129)>IFN $\alpha\beta$ R^{-/-}(129)). Infection with X31 revealed that susceptibility correlated with the ability of stromal cells such as the lung epithelia to respond to IFN $\alpha\beta$ signalling. IFN $\alpha\beta$ R^{-/-}>IFN $\alpha\beta$ R^{-/-} or 129>IFN $\alpha\beta$ R^{-/-} chimeras were resistant to severe X31-induced disease and this correlated to no upregulation of DR5 expression in IFN $\alpha\beta$ R^{-/-} epithelia. Conversely, chimeras where epithelial cells could sense IFN $\alpha\beta$ signalling (129>129 and IFN $\alpha\beta$ R^{-/-}(129)>129) had higher epithelial expression of DR5 compared to epithelia from resistant chimeras and this associated with increased frequency of epithelial cell death and host susceptibility (Figure 24A-B).

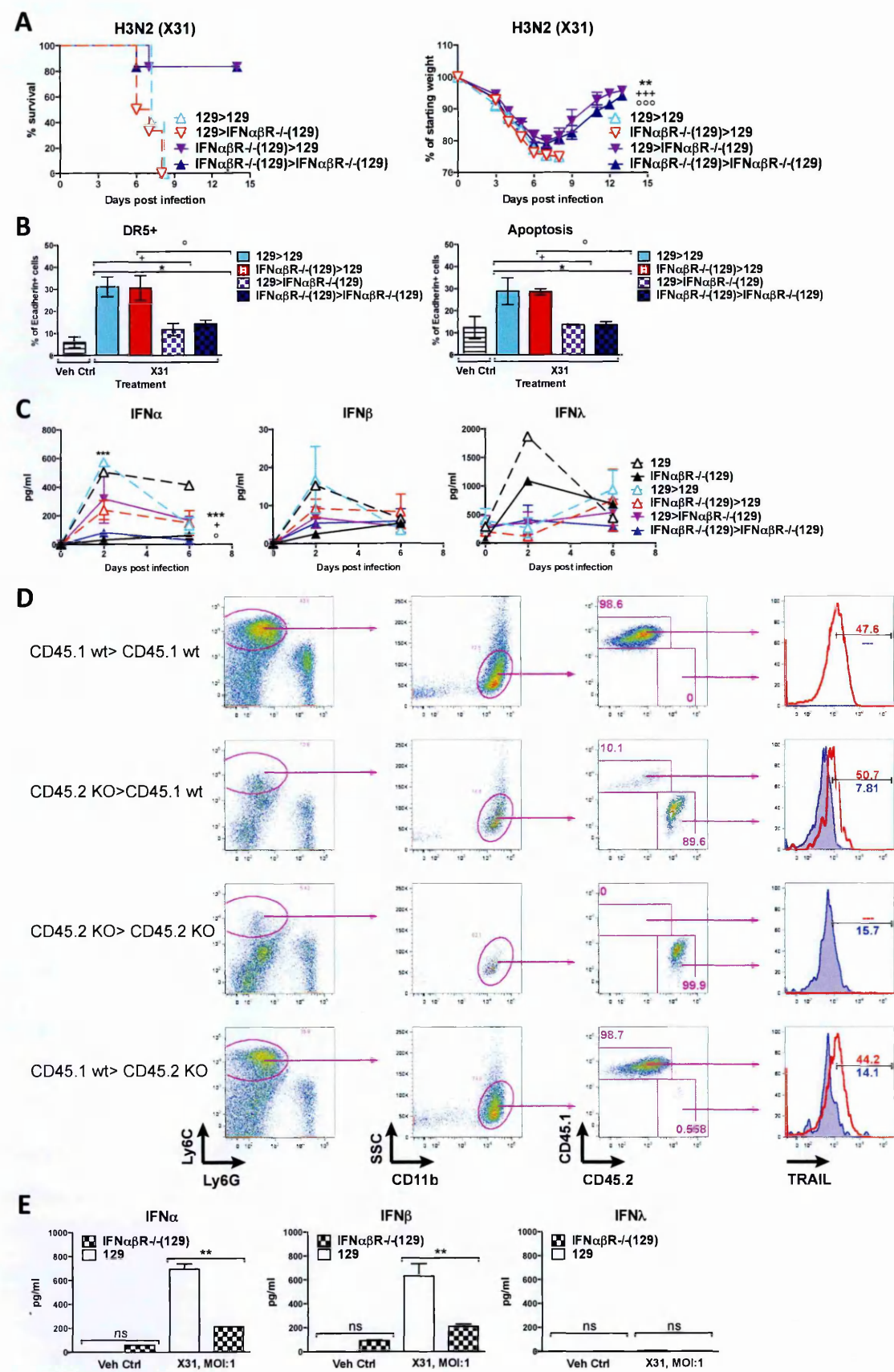
Given the low levels of IFN $\alpha\beta$ seen in BAL fluid from infected whole IFN $\alpha\beta$ R^{-/-}(129) mice and the lack of responsiveness of IFN $\alpha\beta$ R^{-/-}(129) pDCs we were curious to understand where the IFN $\alpha\beta$ signal was coming from in the IFN $\alpha\beta$ R^{-/-}(129)>129 chimera. To assess this, we measured IFN levels throughout infection and observed that IFN α , β and λ levels in the lung during X31 infection of 129>129 and IFN $\alpha\beta$ R^{-/-}(129)>IFN $\alpha\beta$ R^{-/-}(129) chimeras were comparable to the intact mice of the same genotype while both 129>IFN $\alpha\beta$ R^{-/-}(129) and IFN $\alpha\beta$ R^{-/-}(129)>129 chimeras had intermediate levels of IFN α , β and λ (Figure 24C). As it was unclear which cells are the source of IFN $\alpha\beta$ and of the TRAIL-mediated signal required for epithelial cell death in the IFN $\alpha\beta$ R^{-/-}(129)>129 chimeras, we decided to perform an in depth analysis using congenic Wt CD45.1⁺ 129 mice, to allow us to trace the origin of immune cells in the infected lung. In IFN $\alpha\beta$ R^{-/-}(129)>CD45.1⁺ 129 chimeras, we identified a population of AMs or monocytic cells

of host origin (CD45.1+). Similar residual populations were found in the other chimeras. Direct comparison between Wt and IFN $\alpha\beta$ R $^{-/-}$ host origin macrophages and monocytes in these chimeras show that TRAIL levels are higher on the Wt than on the IFN $\alpha\beta$ R $^{-/-}$ cells, confirming that TRAIL upregulation requires an IFN $\alpha\beta$ signal (Figure 24D). Moreover, in vitro stimulation of 129 and IFN $\alpha\beta$ R $^{-/-}$ (129) BM-derived macrophages (BMDMs) with live X31 virus revealed a high level of response from 129 BMDM. We found that, like pDCs, 129 BMDMs produce more IFN than IFN $\alpha\beta$ R $^{-/-}$ (129) BMDMs (Figure 24E), implicating this residual monocyte population as the key contributor to the intermediate levels of type I IFNs recorded in IFN $\alpha\beta$ R $^{-/-}$ (129)>129 chimeras. Therefore, radioresistant host monocytes or AMs are a possible source both of IFN $\alpha\beta$ and of TRAIL in IFN $\alpha\beta$ R $^{-/-}$ (129)>129 chimeras.

Taken together, these results delineate a pathway possibly designed to clear infected cells during IAV infection that, if over stimulated, can lead to destruction of the lung epithelial layer and therefore host pathology. We have shown both TRAIL and DR5 expression to be IFN $\alpha\beta$ dependent, and our results strongly indicate that the high levels of pDC derived IFN $\alpha\beta$ observed in 129 lungs throughout IAV infection lead to excessive TRAIL/DR5 interaction; indeed blockade of DR5 and TRAIL interaction protected 129 mice from severe IAV-induced disease. To extend this data and demonstrate that this cascade of events is not specific to 129 mice we performed in-depth analysis of the immune response in another high IFN responding mouse strain: DBA/1 mice.

Figure 4E-F shows that like the 129 strain, DBA/1 mice have high levels of IFN $\alpha\beta$ in the BAL fluid during infection and this correlated to severe disease. To

Figure 24



ascertain whether or not the DBA/1 strain recapitulated the 129 phenotype we assessed cellular recruitment and expression of TRAIL and DR5 throughout X31 infection by flow cytometry. As was observed in the 129 strain pDCs, NK cells and IMcs are recruited in elevated numbers to DBA/1 lungs (Figure 25A). Furthermore, TRAIL expression on IMcs and DR5 expression on epithelial cells in DBA/1 mice was comparable to what was observed in 129s and markedly higher than what was recorded from the B6 strain (Figure 25B). Finally, depletion of Gr-1⁺ cells (pDCs, IMc and neutrophils) or mAb blockade of TRAIL signalling resulted in an increase in resistance to X31-induced disease in DBA/1 mice, although this was not statistically significant (Figure 25C). We therefore conclude that the IFN $\alpha\beta$ -driven inflammation leading to immunopathology is a general phenomenon observable across a wide range of mouse models of IAV infection.

Figure 24: X31 induced DR5 expression on 129 lung epithelial cells and subsequent host pathology is dependent upon IFN $\alpha\beta$ signalling. (A-D) BM chimeras were generated: 129>129 (light blue), IFN $\alpha\beta$ R^{-/-}-(129)>129 (red), 129>IFN $\alpha\beta$ R^{-/-}-(129) (purple) and IFN $\alpha\beta$ R^{-/-}-(129)>IFN $\alpha\beta$ R^{-/-}-(129) (dark blue) and infected with X31 (800TCID₅₀) and (A) weight loss and survival was recorded. (B) Expression of DR5 on epithelial cells and epithelial cell death was assessed at 7dpi. (C) IFN concentrations during X31 infection in BAL from bone marrow chimeric mice and 129 (open triangles) and IFN $\alpha\beta$ R^{-/-} (black triangles) was measured by ELISA. (D) At 6dpi lung single cell suspensions were prepared, and expression of TRAIL on inflammatory monocytes was assessed by flow cytometry. Gating strategy depicted using Flow Jo Version 9.5, red histograms indicate CD45.1⁺ Wt 129 cells and blue indicate IFN $\alpha\beta$ R^{-/-}-(129) CD45.2⁺ cells. (E) Bone Marrow derived macrophages from 129 and IFN $\alpha\beta$ R^{-/-}-(129) mice were stimulated with X31, MOI of 1 for 24hrs, concentrations of IFNs in supernatants were then assessed by ELISA. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=3-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss and IFN ELISAs), by Mann Whitney test (DR5 expression and frequency of apoptotic epithelial cells) or Log-rank (Mantel-Cox) Test (survival), 2-way ANOVA with Bonferroni post tests where * denotes 129>129:IFN $\alpha\beta$ R^{-/-}->IFN $\alpha\beta$ R^{-/-}-, + for 129>129:129 IFN $\alpha\beta$ R^{-/-}-, and ° represents IFN $\alpha\beta$ R^{-/-}->IFN $\alpha\beta$ R^{-/-}:-IFN $\alpha\beta$ R^{-/-}->129. Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

Figure 25

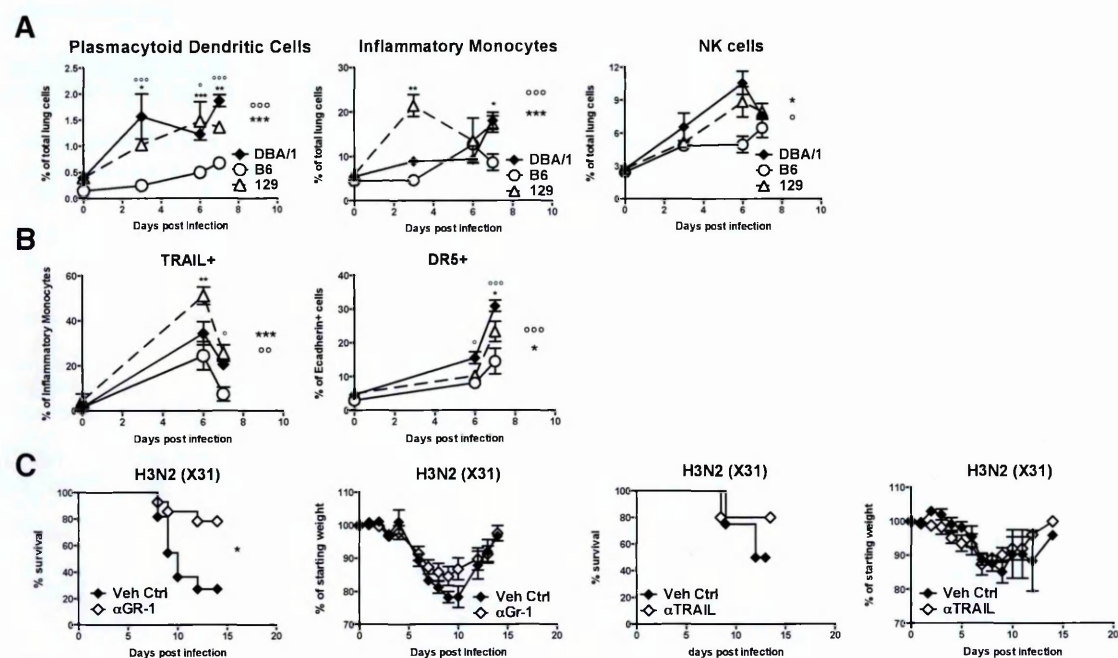


Figure 25: DBA/1 strain susceptibility to influenza induced disease follows high type I IFN production, pDC and iMC recruitment and TRAIL/DR5 expression. 129 (open triangles), B6 (open circles), and DBA/1 (black diamonds) mice were infected i.n with X31 (800TCID₅₀). (A-B) At specified time points, lung single cell suspensions were prepared, (A) recruitment of pDCs, NK cells and IMCs and (B) expression of TRAIL on IMCs and DR5 on epithelial cells was assessed by flow cytometry. DBA/1 mice were infected with X31 and treated with (C) α Gr-1 (open diamonds) or Veh ctrl (black diamonds) or (D) α TRAIL (open diamonds) or Veh ctrl 24hrs before infection with X31. α Gr-1 treatment was continued every 48hrs thereafter and α TRAIL treatment every 24hrs, all treatments were ceased at 12dpi. Weight loss and mortality was recorded throughout infection. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where $n=2-6$, except for (C) where data is pooled from two experiments ($n=15$). B6:129 *, and B6: DBA/1: °, where *** $p<0.0001$, ** $p<0.001$, * $p<0.01$ by 2-way ANOVA with Bonferroni post tests (weight loss, cellular recruitment, and TRAIL and DR5 expression) or Log-rank (Mantel-Cox) Test (survival). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and symbols above individual points indicate significance as assessed by Bonferroni post test.

3.4 Discussion

Severe influenza induced disease is characterised by the rapid development of acute lung injury associated with a vigorous inflammatory response (Louie et al., 2009; Mauad et al., 2010; Peiris et al., 2004). However, severity of disease significantly differs between individuals, and this variance has been hypothesised to be in part due to genetically determined host factors. Here, we demonstrate that host-intrinsic differences can determine the severity of influenza induced disease and that responsiveness to IFN $\alpha\beta$ signalling is a host-specific determinant with protective or detrimental potential. High susceptibility of the mouse strains 129, DBA/1, CBA/J and DBA/2 correlated to significantly higher levels of IFN $\alpha\beta$ in the BAL fluid, as compared to more resistant B6 and BALB/C mice. Further comparison of 129 and B6 mice revealed higher resting numbers of potent IFN producing pDCs in 129 mice, which were more robustly recruited to the infected lungs. Excessive pDC presence in the infected lung and their subsequent secretion of IFN $\alpha\beta$ was found to be upstream of a robust proinflammatory cytokine and chemokine response as well as recruitment of innate inflammatory cells such as IMcs in 129 lungs. Significantly, IFN $\alpha\beta$ signalling induced the upregulation of TRAIL on IMcs and the TRAIL receptor DR5 on lung epithelia, and this correlated to higher numbers of apoptotic epithelial cells in 129 mice. Thus, excessive IFN $\alpha\beta$ induced pronounced epithelial cell death and an exaggerated proinflammatory response that ultimately led to morbidity and mortality of the 129 strains (Figure 26).

Historically, IFN $\alpha\beta$ is thought to be exclusively protective during viral infections. However, data presented in this chapter argue that protection afforded by IFN $\alpha\beta$ is a function of concentration. As exquisitely demonstrated in the B6

Figure 26

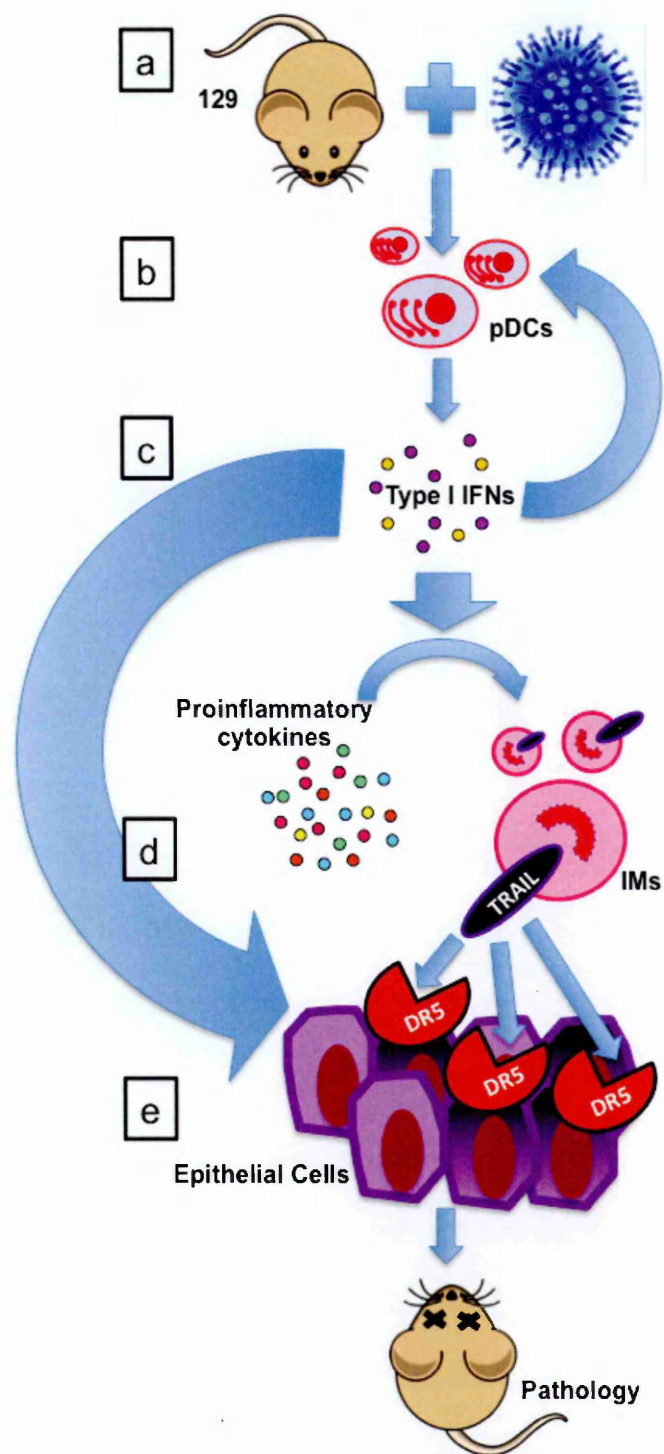


Figure 26: Graphic representation of hypothesized mechanism of 129 pathology. (a) Influenza infection of 129 mice. (b) Highly responsive 129 pDCs are recruited to the infected lung where they secrete large amounts of type I IFNs. (c) IFN $\alpha\beta$ induces secretion of proinflammatory cytokines and chemokines and the recruitment of innate inflammatory cells such as IMcs. (d) IFN $\alpha\beta$ signalling also induces upregulation of TRAIL on IMs and DR5 on epithelial cells. (e) Interaction between TRAIL on IMs and DR5 on epithelial cells leads to epithelial cell death, therefore severe lung damage and host pathology.

strain, too much or too little IFN $\alpha\beta$ signalling can lead to increased morbidity. Similar results were obtained by Beilharz et al., where moderate oral doses of IFN α were protective during influenza challenge, while animals given high doses of IFN α suffered higher morbidity than placebo controls, even though the treatment in this study lasted for 10 days and therefore, immediate effects of IFN treatment could not be distinguished from long-term effect (Beilharz et al., 2007). Furthermore, a backcrossing study using IAV resistant B6 mice and IAV susceptible DBA/1 mice demonstrated a host specific, positive correlation between IFN $\alpha\beta$ and other proinflammatory cytokine expression with disease severity (Boon et al., 2009). A delicate balance must therefore be struck, where type I IFN signalling must be sufficient to induce an adequate immune response yet not so overreaching as to induce immunopathology.

To our knowledge this is the first study to demonstrate IFN $\alpha\beta$ directly contributing to severe disease in IAV infection. However, IAV is not the only virus to have a complex relationship with IFN $\alpha\beta$. A recent study conducted by Wetzel and others (2014) demonstrated that elevated levels of IFN β in lungs of B6 mice infected with Sendai virus directly correlated with increased host morbidity and mortality. Notably, this pathogenic potential of IFN $\alpha\beta$ was revealed only in the absence of the ISG Ifit2, a gene that codes for a potent restriction factor of Sendai virus replication (Wetzel et al., 2014). Analogously, the experiments presented in this chapter have been performed in inbred laboratory mouse strains whose genomes do not code for the functional IAV restricting ISG Mx1 (Pavlovic et al., 1995). Whether or not exaggerated IFN $\alpha\beta$ signalling retains its pathogenic potential on an Mx1 functional background will be addressed in a later chapter. It is however interesting to note that Wetzel et al.'s study was performed in B6 mice,

a strain we identify as low IFN $\alpha\beta$ producers, while in an earlier study using a 129 mice, Lopez et al. recorded lower weight loss in IFN $\alpha\beta$ R $^{-/-}$ (129) mice than their wild-type counterparts during Sendai virus infection (Lopez et al., 2006). Furthermore, Shin et al. recently showed that reconstituting Mx1 protein functionality through congenic breeding on the DBA/2 background did not confer increased resistance to PR8 induced disease compared to Mx1 non functional DBA/2 controls (Shin et al., 2015). In our own study we class the DBA/2 strain as a high IFN producing background and similar to what we observed in 129 mice, IAV induced pathology of DBA/2 mice that express functional Mx1 associated with elevated levels of a myriad of proinflammatory cytokines including IP-10, IL-6 and MCP-1, as compared to Mx functional B6 mice. However, in contrast to our results, expression of IFN β mRNA at 3dpi in the lung was comparable between DBA/2 and B6 background and ultimately the authors attribute Mx1 functional DBA/2 mice susceptibility to IAV replication out pacing production of Mx1 protein in the DBA/2 but not the B6 background (Shin et al., 2015). Given that the assessment of IFN $\alpha\beta$ production in the lung was limited to IFN β mRNA and at only one time point, it would be interesting to compare concentrations of IFN $\alpha\beta$ over the entire time course of this study and to determine whether or not blockade of IFN $\alpha\beta$ signalling could ameliorate disease, as seen in our results. Collectively, this data gives credence to the idea that even in the context of potent antiviral ISGs, high levels of type I IFNs can still drive immunopathology.

While IFN $\alpha\beta$ has been shown to be protective by induction of antiviral factors early in Lymphocytic choriomeningitis virus (LCMV) infection (Muller et al., 1994), blockade of type I IFN signalling late in infection has been shown to increase LCMV clearance. One study suggested that this disparity in IFN $\alpha\beta$ action

was due to IFN $\alpha\beta$ blocking IFN γ expression, while promoting IL-10 secretion. A parallel study indicated that type I IFN signalling drove expression of programmed cell death 1 ligand (PD-L1) on dendritic cells, antagonized expansion of T cells, B cells, NK cells, and macrophages, and was associated with splenic architecture disorganization. Ultimately, both studies concluded type I IFN-mediated dampening of the immune response impaired CD4⁺ T cell function and thereby supported viral persistence (Teijaro et al., 2013; Wilson et al., 2013). IFN γ secretion and CD4⁺ T cell recruitment was not blunted in 129 mice, compared to IFN $\alpha\beta$ R^{-/-}(129) mice, however IL-10 was massively induced in 129 lungs. Concurrently, IFN $\alpha\beta$ has been shown to induce IL-10 secretion in many disease settings (Chang et al., 2007a; McNab et al., 2014; Zhang et al., 2011). The high concentrations of IL-10 found in 129 lungs could be downstream of IFN $\alpha\beta$ signalling. However, levels of IL-10 late in infection IFN $\alpha\beta$ R^{-/-}(B6) mice were elevated compared to levels found in Wt B6 mice, demonstrating that IL-10 is not only induced by IFN $\alpha\beta$. Indeed, as IL-10's primary function is to limit immunopathology it is also secreted in response to other proinflammatory cytokines and is downstream of TLR activation (Couper et al., 2008). 129 and IFN α R^{-/-}(B6) mice both experience heavier disease burden than their IFN $\alpha\beta$ R^{-/-} or Wt counterpart (respectively) demonstrating that induction of IL-10 in this model is not driven by IFN $\alpha\beta$ alone.

B cell recruitment to 129 lungs was markedly reduced, which correlated to a trend for lower induction of IAV specific antibodies. Price et al. have previously demonstrated that IFN $\alpha\beta$ R deficiency on the 129 background allowed for better induction of IAV specific antibodies (Price et al.). Interestingly, this depression of B cells by IFN $\alpha\beta$ seems to be 129 specific, as it was not observed on the B6

background. Although we conclude IFN $\alpha\beta$ mediated immunopathology in 129 mice occurs independent of adaptive immunity, it is interesting to note that titres of X31 in 129 lungs persist longer than what was observed in IFN $\alpha\beta$ R $^{-/-}$ (129) lungs. Perhaps this is linked to the lower antibody response. This is significant, as persistence of viral particles in the lung late in infection can continue to stimulate the immune system, facilitating prolonged IFN $\alpha\beta$ signalling and in this way contribute to 129 pathology.

Excessive type I IFN signalling may not only associate with host pathology during acute virus infection but is also observed chronic viral infections. For example, chronic production of IFN α by human pDCs during Human Immunodeficiency Virus (HIV) infection has been proposed to contribute to persistent immune activation (O'Brien et al., 2013; Rajasuriar et al., 2013), while Strydom et al. demonstrated that pDC derived IFN α led to expression of TRAIL on pDCs and CD4 $^{+}$ T cells and concomitant death receptors on CD4 $^{+}$ T cells; this interaction induced apoptosis of uninfected CD4 $^{+}$ T cells and consequently severe host pathology (Strydom et al., 2009). An earlier in vitro study demonstrated that IFN α produced by pDCs after HIV-induced TLR7 stimulation was responsible for TRAIL expression (Hardy et al., 2007). Furthermore, ex vivo analysis of CD8 $^{+}$ T cells from HIV positive individuals found that those categorised with a nonprogressive disease phenotype (ie: resistant to severe virus induced disease) exhibited lower expression of interferon-stimulated genes (Herbeuval et al., 2006). However, like in our own study, the role of IFN $\alpha\beta$ in HIV infection is a fine balance; host intrinsic defects in pDCs, particularly relating to decreased ability to produce type I IFN, have been associated with enhanced HIV replication (O'Brien et al., 2013; Siegal et al., 1999; Soumelis et al., 2001). Furthermore, administration of an

antagonist for IFN $\alpha\beta$ R exacerbated disease in a pathogenic SIV rhesus macaque model, while treatment with pegylated-IFN α 2a in the same model before and during viral challenge reduced the frequency of viral transmission (Sandler et al., 2014). Additionally, 2 studies conducted in HIV infected humans treated with IFN α 2b demonstrated that treatment led to a transient decrease in viral load (Asmuth et al., 2010; Lane et al., 1990). Further study on IFN α 2b treated HIV infected individuals found that expression levels of a set of canonical ISGs, including a potent HIV restrictor: Mx2, correlated to reduction in viral load (Hubbard et al., 2012). Thus, similar to IAV infection, IFN $\alpha\beta$ antiviral effects are vital for control of virus replication, many potent HIV restricting factors such as Mx2 and tetherin are ISGs and therefore IFN $\alpha\beta$ is integral to host protection from HIV, however inflammation driven by IFN $\alpha\beta$ has the potential to drive pathology, in this case through contribution to CD4⁺ T cell death.

In light of its well-established anti-viral role it is surprising that loss of type I IFN signalling in 129 mice does not increase host susceptibility to influenza induced disease. We hypothesise that IFN $\alpha\beta$ R^{-/-}(129) mice are able to control influenza replication through the presence of a second, entirely independent, yet redundant IFN system: the type III IFNs (Kotenko et al., 2003; Mordstein et al., 2008; Sheppard et al., 2003). Concentrations of IFN λ found in IFN $\alpha\beta$ R^{-/-}(129) BAL are analogous to those found in resistant B6 lungs during influenza infection. Furthermore, Crotta et al confirmed ISG induction and control of influenza replication in cultured IFN $\alpha\beta$ R^{-/-} and Wt AECs is equivalent (Crotta et al., 2013). Thus, the antiviral response elicited by IFN λ is sufficient to protect IFN $\alpha\beta$ R^{-/-}(129) mice. By the same token, it is therefore surprising that IFN $\alpha\beta$ R^{-/-}(B6) mice are more susceptible than their Wt controls, as an intact IFN λ pathway should be

sufficient to protect mice from influenza induced disease. However, levels of IFN λ in IFN $\alpha\beta$ R $^{-/-}$ (B6) BAL are significantly lower than all other mouse strains tested. This indicates that without IFN $\alpha\beta$ signalling, IFN λ induction and or signalling may be in some way impaired.

IFN λ has been demonstrated to act in an autocrine and paracrine manner to amplify its own production, independent of IFN $\alpha\beta$ signalling (Ank et al., 2006b). However, mAb blockade or genetic deletion of IFN $\alpha\beta$ R on pDCs in this study blunted their secretion of not only IFN α and but also IFN λ in response to stimulation with X31. Furthermore, genetic deletion of type I IFN signalling in both 129 and B6 mice decreased pulmonary levels of IFN λ during X31 infection. In contrast to IFN α and IFN λ , IFN β levels were not blunted by mAb blockade of IFN $\alpha\beta$ R signalling on 129 pDCs, but whole lung levels of IFN β in IFN $\alpha\beta$ R $^{-/-}$ (129) mice were reduced compared to levels observed in 129 lungs. It has been reported that IFN $\alpha\beta$ production by pDCs does not depend on presence of a functional IFN $\alpha\beta$ R (Barchet et al., 2002). We observe an essential requirement of IFN $\alpha\beta$ R for not only IFN α but also IFN λ production in 129 pDCs, only IFN β induction appears independent of IFN $\alpha\beta$ R signalling. Additionally, this appears to be cell type intrinsic, as expression of IL-28A mRNA was comparable between Wt, IFN $\alpha\beta$ R $^{-/-}$ (129) and STAT1 $^{-/-}$ (129) AECs (Figure 9). Similarly, Crotta et al. also observed that IL-28A gene induction by IAV was not impeded by deficiency of both IFN $\alpha\beta$ R and IFN λ R in AECs (Crotta et al., 2013). Although pDCs are potent producers of IFN λ invitro, depletion of this cell type and other PDCA-1+ cells from 129 mice revealed that, unlike most other cytokines assessed, concentrations of IFN λ in the BAL remained high, indicating there are other potent cellular sources of IFN λ . As AEC upregulation of IL-28A mRNA is not negatively impacted by IFN $\alpha\beta$ R

deficiency, in vivo secretion of IFN λ by this cell type is unlikely to be affected by lowered pulmonary levels of IFN α . Alternatively, CD8 α^+ DCs have also been demonstrated to be potent IFN λ producers and may therefore also contribute to pulmonary levels of IFN λ in α PDCA-1 treated 129 mice (Crotta et al., 2013; Lauterbach et al., 2010). In addition, IFN β levels in α PDCA-1 treated 129 mice were relatively unaffected and therefore IFN β may contribute to the propagation of IFN λ induction. Yet, the question remains why IFN $\alpha\beta$ R deficiency negatively impacts on IFN λ response so markedly in IFN $\alpha\beta$ R $^{-/-}$ mice. It is possible that a downstream signalling molecule, common to both IFN $\alpha\beta$ and IFN λ , requires a tonic type I IFN signal to maintain adequate expression levels. Previous studies have demonstrated that members of the STAT and IRF families require low-level IFN β signalling to maintain basal levels (Fleetwood et al., 2009; Gough et al., 2010). Thus, while depletion of PDCA-1 $^+$ cells only decreases type I IFN signalling during influenza infection, complete life time ablation of IFN $\alpha\beta$ signalling (as in IFN $\alpha\beta$ R $^{-/-}$ mice) may result in diminished basal expression of this specific factor and therefore may compromise downstream biological responses such as amplification of IFN λ . It is also conceivable that this molecule is more abundant, or more readily activated in the 129 background, therefore explaining why loss of a tonic IFN $\alpha\beta$ signal in IFN $\alpha\beta$ R $^{-/-}$ (129) mice leads to a less dramatic reduction of IFN λ than that seen in the IFN $\alpha\beta$ R $^{-/-}$ (B6) BAL.

Although IFN λ induces the same downstream effects as IFN $\alpha\beta$, it is of significant interest that the tissue distribution of IFN $\alpha\beta$ R and IFN λ R are not equivalent. While IFN $\alpha\beta$ R is ubiquitously expressed, IFN λ R expression tends to be restricted to epithelial cell surfaces (Mordstein et al., 2008; Sheppard et al., 2003; Sommereyns et al., 2008). Type I IFNs therefore may play a nonredundant role in

activating immune cells, which is sufficient to induce protection on the B6 background, yet exaggerated in Wt 129s leading to host immunopathology. Loss of IFN $\alpha\beta$ immune cell activation is countered by sufficient concentrations IFN λ to protect the lung epithelia in IFN $\alpha\beta$ R $^{-/-}$ (129) mice, however lower IFN λ levels seen in IFN $\alpha\beta$ R $^{-/-}$ (B6) lungs are less effective. This would also explain the difference between the data presented in this chapter and a previous study by Garcia-Sastre et al. that also employed IFN $\alpha\beta$ R $^{-/-}$ mice on the 129 background. In contrast to the results presented here, Garcia-Sastre et al. reported an increased susceptibility to IAV in IFN $\alpha\beta$ R $^{-/-}$ (129) compared to their Wt counterparts. The key difference between Garcia-Sastre et al.'s study and our own is the IAV strain used. Unlike our own study which used 3 archetypal IAV strains, Garcia-Sastre et al. employed A/WSN/33 IAV strain, that unlike other strains of IAV can also infect neurons (Garcia-Sastre et al., 1998a). Neurons are insensitive to IFN λ (Sheppard et al.) and thus in this setting, IFN $\alpha\beta$ R is crucial for ISG induction, consequent control of IAV replication and ultimately, host protection.

Higher pDC frequencies observed in naïve 129 mice are not seen in IFN $\alpha\beta$ R deficient mice of the same strain. The reduction of IFN $\alpha\beta$ and IFN λ recorded in the pulmonary environment of IFN $\alpha\beta$ R $^{-/-}$ mice during X31 infection may be a result of lower pDC numbers and an ablation in their functionality. In some settings IFN α has been demonstrated to be an autocrine survival factor for the normally labile pDCs (Ito et al., 2001; Kadowaki et al., 2000), while in other situations IFN $\alpha\beta$ R induces caspase 1 mediated pDC death (Swiecki et al., 2011). Although not investigated in depth, persistent higher numbers of pDCs in 129 lungs suggests that high IFN $\alpha\beta$ signalling does not promote apoptosis of this cell type in IAV infection or, alternatively it is possible that IFN $\alpha\beta$ R signalling preferentially drives

pDC recruitment and proliferation in addition to apoptosis, thereby maintaining high numbers of this cell type. Chen et al. demonstrated that generation of pDCs from common lymphoid progenitors (CLPs) involves positive feedback between IFN α β R and Fms-like tyrosine kinase 3 (Flt3). Flt3 ligand (Flt3L) induces secretion of IFN α β from CLPs that in turn, induces Flt3 up-regulation, thereby facilitating survival and proliferation of CLPs, as well as differentiation into pDCs (Chen et al., 2013). IFN α β R deficiency may therefore explain lower basal frequencies of IFN α β R^{-/-}(129) pDCs and their abolished responsiveness to viral stimulus. Furthermore, this data is again suggestive of an increased presence of a tonic type I IFN signal in 129 mice that could maintain higher basal numbers of pDCs.

Highly responsive pDCs in 129 lungs are likely to be the primary cellular source of IFN α induced by IAV. Depletion of PDCA-1⁺ cells effectively depleted pDCs in 129 mice and drastically lowered IFN α pulmonary concentrations. However, as PDCA-1 is upregulated by other cell types in response to IFN α β stimulation and IFN α β R engagement induces further secretion IFN α subtypes, it is probable that other cell types contribute to the high levels of IFN α β observed in 129 mice. Evolutionarily, pDCs are likely designed as a secondary defence mechanism to viral infection. Using an IFN α 6 GFP reporter mouse to determine the cellular source of IFN α in NDV Kumagai et al. found that pDCs did not make IFN α in response to pulmonary NDV infection, instead AMs and cDCs were the primary source of IFN α and it was only when AMs were depleted that pDCs secreted IFN α (Kumagai et al., 2009). This would explain why in many viral infections, including IAV, pDCs are dispensable for induction of an IFN α β response and ultimate resolution of infection. In the case of IAV, AMs appear to be the primary source of IFN α β and are sufficient to induce an antiviral state in the lung, thereby impeding

IAV spread (GeurtsvanKessel et al., 2008; Soloff et al., 2012). Although dispensable, pDC secretion of IFN $\alpha\beta$ can be useful if the first defence line is evaded, for example Kallfass et al found that in infection of IFN β GFP reporter mice with a delNS1 strain of IAV, epithelial cells and lung macrophages were the primary expressers of IFN β GFP protein. However, upon infection with the parental NS1 functional strain of IAV, expression of IFN β was blocked in epithelial cells and to a lesser extent in macrophages and instead, other undefined cell types, including CD11c+ cells made up the majority of IFN β + cells (Kallfass et al., 2013). Further characterization of these cell types was not performed in this study, however pDCs are known to be CD11c+. It is therefore possible that in IAV infection, while the primary source of IFN $\alpha\beta$ is AMs, pDCs may offer a supplemental source of IFN $\alpha\beta$ which is unimpeded by NS1 action. Importantly, the above studies were performed in mice of the B6 background it is therefore possible that in 129 mice, pDCs may subvert their role as secondary responders and respond immediately to IAV recognition, and this leads to exaggerated IFN $\alpha\beta$ levels and host pathology.

IFN α levels in the lung during infection were intermediate in the IFN $\alpha\beta$ R-/->129 and 129>IFN $\alpha\beta$ R-/- chimeras, compared to the higher concentrations found in 129>129 and significantly lower levels in IFN $\alpha\beta$ R-/->IFN $\alpha\beta$ R-/- chimeras. This is in contrast to data collected from whole IFN $\alpha\beta$ R-/- (129) where pulmonary concentrations of IFN α were almost never detected at the time points assessed. Taken with the results gleaned from in vitro stimulation of Wt pDCs (both 129 and B6), we hypothesise that the cellular source of IFN α during IAV infection is dependent on IFN $\alpha\beta$ R positive feedback loop. IFN β levels were decreased in both IFN α R-/- (129) and to a lesser extent α PDCA-1 treated mice so the comparable levels of this cytokine in all chimeras is in line with previous data presented in this

chapter. Thus the intermediate levels in the IFN α β R-/-129 chimera are surprising, however the presence of a radiation resistant monocyte population provides an immune source of IFN α that may compensate for the lack of positive feedback in the rest of the immune compartment. These radioresistant cells may upregulate PDCA-1 upon stimulation with IFN α β and therefore would have been depleted in α PDCA-1 treated mice.

It is interesting to note that IFN α levels in the lung during IAV infection clearly correlated with disease severity, yet the link between pathology and pulmonary concentration of IFN β was less convincing. Differences in IFN β levels in the lung between susceptible 129 and resistant B6 strain were consistently less impressive than what was observed in concentrations of IFN α and depletion of PDCA-1+ cell types did not massively impact on IFN β concentrations in the lung during IAV, yet did protect 129 mice from severe disease. This may be a reflection of IFN β expression being tightly controlled and only achieved through direct PRR engagement, while expression of IFN α subtypes (which are indiscriminately read out in the IFN α ELISA used in this study) are induced in some cell types such as pDCs directly downstream of pathogen recognition and further amplified by ligand binding to IFN α β R. It would be interesting to disentangle IFN β and IFN α mediated effects on host immunity to IAV and it is possible that it is only the downstream amplification of IFN α , which is so pronounced in 129 mice, that drives pathology and genetic ablation of all IFN α subtypes could be sufficient to protect the 129 strain from immunopathology.

High proinflammatory cytokine production during influenza infection is commonly associated with IAV disease severity. PDCA-1+ cell depletion or genetic ablation of IFN α β R signalling in 129 mice renders proinflammatory cytokine

concentrations equivalent to levels seen in the resistant B6 strain. However, in vitro stimulation of 129 pDCs revealed that, with the exception of Mip-1 β , the pro-inflammatory cytokines that are increased in influenza-infected 129 lungs are not produced by pDCs, suggesting that the augmented pro-inflammatory cytokine milieu found in infected 129 mice is indirectly induced by upstream, host intrinsic IFN $\alpha\beta$ responsiveness. Further investigation is required to identify which cell types IFN $\alpha\beta$ stimulates to induce the release of MCP-1, IL-6, IP-10, IFN γ , Eotaxin, G-CSF, and perhaps Mip-1 β . AMs and IMcs are known to secrete many proinflammatory mediators in response to IAV making them likely sources (Cheung et al., 2002; Seo et al., 2011; Woo et al., 2010). Additionally, Teijaro et al. reported that lung endothelial cells secrete similar proinflammatory cytokines during IAV infection in a partially IFN $\alpha\beta$ dependent manner, making them interesting candidates for future research (Teijaro et al.). It is important to note that although levels of proinflammatory cytokines recorded in 129 lungs were monumentally higher than concentrations observed in IFN $\alpha\beta$ R $^{-/-}$ (129), B6 and IFN $\alpha\beta$ R $^{-/-}$ (B6) lungs, these cytokines were still induced above baseline in response to IAV infection in the more resistant mouse strains. Indeed in some cases (Eotaxin, G-CSF, IFN γ and IL-6) levels of proinflammatory cytokines were higher in IFN $\alpha\beta$ R $^{-/-}$ (B6) lungs than concentrations observed in B6 Wt lungs, demonstrating that proinflammatory cytokine induction is not just dependent upon IFN $\alpha\beta$ R signalling but is also a function of viral replication and disease burden.

IFN $\alpha\beta$ -promoted MCP-1 secretion in the lung induces recruitment of IMcs. IMc escape from the bone marrow is entirely dependent upon CCR2 engagement, MCP-1 being a primary ligand for CCR2 (Kuziel et al., 1997; Serbina and Pamer,

2006). AECs secrete MCP-1 upon IAV infection to initially recruit monocytic cell types (Herold et al., 2008), furthermore IFN $\alpha\beta$ action on IMcs themselves has been shown to further propagate IMc recruitment during IAV infection by preferentially inducing secretion of MCP-1 from this cell type (Skountzou et al.). This cascade of IFN $\alpha\beta$ signalling, MCP-1 secretion, IMc recruitment and their subsequent IFN $\alpha\beta$ mediated upregulation of TRAIL further demonstrates IFN $\alpha\beta$ pathogenic potential. However, advances in delineating monocyte subtypes have revealed a myriad of myeloid populations that are not only dependent upon CCR2 recruitment, but can express TRAIL during IAV infection (Ellis et al., 2015; Langlet et al., 2012; Misharin et al., 2013). It is therefore likely that beyond our stringently defined Ly6C^{hi}, CD11b⁺, CD11c⁻, FSC^{int}, SSC^{int} IMcs, other monocyte-derived populations contribute to TRAIL mediated tissue destruction in this model. Indeed, Herold et al. identified a CCR2 dependent F4/80⁺ Gr-1^{int}, CD11c^{int} population that they termed exudate macrophages. Like IMcs in this study, exudate macrophages upregulated TRAIL during severe IAV infection and thereby induced AEC apoptosis (Herold et al., 2008). However Herold et al. only observed TRAIL expression on exudate macrophages in the context of PR8 and not X31 infection, which is in contrast to the TRAIL⁺ IMc population we observed in X31 infected B6 mice in Figure 23. This could be due to differences in experimental models: Virus dose and route and volume of inoculum were different between studies and as X31 induced weight loss was not reported, it is difficult to understand whether or not our infection of B6 mice with X31 is comparable. These differences notwithstanding, this study focused on TRAIL expressing Gr-1^{int} cells, which potentially excludes Ly6C^{hi} IMcs reported in this study and given the comparatively lower frequency of AEC apoptosis observed by us in X31 infected B6 mice it is possible that TRAIL induced

apoptosis of AECs was indistinguishable from other forms of cell death that occurs during IAV infection in Herold et al.'s study. In a follow up study this group demonstrated both exudate macrophages and AMs express TRAIL in IAV infection and this was dependent upon autocrine IFN $\alpha\beta$ signalling. Generation of bone marrow chimeras where the hematopoietic system was deficient for IFN $\alpha\beta$ R or TRAIL lowered AEC apoptosis in B6 mice infected with PR8 and chimeras with a TRAIL $^{-/-}$ hematopoietic system were protected from IAV induced morbidity (Hogner et al., 2013). These studies are largely in agreement with what we have reported in this chapter: monocyte and AM populations express TRAIL in response to IFN $\alpha\beta$ signalling during IAV infection and thereby induce AEC apoptosis. Yet AMs are radioresistant (Kennedy and Abkowitz, 1997), as we observed in our IFN $\alpha\beta$ R $^{-/-}$ (129) >129 chimeras, where the IFN $\alpha\beta$ dependent TRAIL signal to induce AEC death and therefore host mortality was supplied by the residual host monocyte or AM population (Figure 24). In contrast, Hogner et al observed AM expression of TRAIL was entirely ablated in chimeras made with IFN $\alpha\beta$ R $^{-/-}$ or TRAIL $^{-/-}$ bone marrow and this correlated to reduced frequencies of apoptotic AECs. This discrepancy between Hogner et al.'s and our own study may be due to the levels of IFN $\alpha\beta$ during infection in each mouse strain used. Hogner et al. employed B6 mice and as observed in our study, this strain has a comparatively lower propensity to make IFN $\alpha\beta$ than the 129 strain used in our bone marrow chimera experiments. Furthermore, IFN $\alpha\beta$ secretion is markedly impeded by IFN $\alpha\beta$ R deficiency, therefore amplification of IFN $\alpha\beta$ signalling in B6 AMs may be more severely affected than in 129 AMs. Coupled with this, prolonged IFN $\alpha\beta$ R engagement is required for induction of TRAIL (Kalie et al., 2008). Thus apoptosis of AECs by TRAIL $^{+}$ residual host AMs may not have been observed by Hogner et al.

as the IFN $\alpha\beta$ signal was insufficient. Differences in these results may also be due to other strain intrinsic features of 129 and B6 mice, beyond IFN $\alpha\beta$ induction of TRAIL.

TRAIL expression by pDCs in response to both IFN $\alpha\beta$ stimulation and IAV recognition has also been observed. In a pDC cell line (GEN2.2) and pDCs isolated from PBMCs, live IAV, TLR7 and 9 agonists all elicited TRAIL upregulation (Chaperot et al., 2006). Balzarolo et al further demonstrated that TLR and IFN $\alpha\beta$ signalling were both required in human pDCs for full TRAIL expression (Balzarolo et al., 2012). Additionally, TRAIL expression was also detected on NK cells as well as CD4+ and CD8+ T cells in IAV infection (Hamada et al., 2013; Ishikawa et al., 2005). However, we did not observe TRAIL on any of these cell types in this study. This may be due to the limited number of time points assessed: NK cell expression of TRAIL was generally found as early as 5dpi, while TRAIL expression on T cells was observed as late as 8dpi. Differences in experimental models such as mouse or IAV strain or disease burden may also contribute to the differing results between these studies and ours.

Modern histopathological analysis of autopsy samples from human H1N1 1918 influenza infection revealed massive lung damage involving significant destruction of the respiratory epithelium (Kash et al., 2006; Taubenberger and Morens, 2006). Significantly, analysis patients hospitalized due to SO-IV H1N1 infection found that expression levels of TRAIL were markedly increased in infected patients over healthy controls (Hogner et al., 2013; Li et al., 2010). X31 induced pathology in 129 mice is characterised by prolonged inflammation and upregulation of TRAIL on IMCs and its receptor DR5 on epithelial cells. Previous studies have demonstrated that DR5 can be upregulated in response to IFN $\alpha\beta$

stimulation (Bernardo et al., 2013), indeed during influenza infection we observed Wt, both 129 and B6 epithelial cells expressed higher levels of DR5, compared to their IFN $\alpha\beta$ R deficient counterparts, strongly indicating DR5 upregulation is downstream of IFN $\alpha\beta$ R signalling and not a function of disease severity. IFN $\alpha\beta$ mediated upregulation of DR5 is associated with higher frequencies of apoptotic epithelial cells late in infection. Generation of bone marrow chimeras confirmed that susceptibility to influenza-induced disease was dependent upon nonhaematopoietic cells such as epithelial cells receiving a type I IFN signal. Mice with IFN $\alpha\beta$ R $^{+/-}$ stroma upregulated DR5 expression on epithelial cells and again this associated with increased frequency of epithelial cell death.

Crotta et.al. demonstrate that induction of ISGs in IFN $\alpha\beta$ R $^{-/-}$, IFN λ R $^{-/-}$ and Wt tracheal epithelial cell cultures is identical meaning that, in this cell type no subset of ISGs is specifically regulated by IFN $\alpha\beta$ or IFN λ . Additionally, we did not observe a change in expression of DR5 in in vitro AEC cultures during influenza infection (data not shown), and Hogner et al. found that direct stimulation of AEC cultures with IFN β did not induce DR5 expression (Hogner et al., 2013). These data appear contrary to our own in vivo data where DR5 expression on epithelial cells is specifically modulated by IFN $\alpha\beta$ signalling. IFN $\alpha\beta$ may therefore act in concert with another factor (e.g.: IAV infection itself or another proinflammatory cytokine) to induce DR5 upregulation and in an in vitro culture system this factor may not be present. Alternatively, lung endothelial cells have been shown to vigorously respond to type I IFN signalling (Teijaro et al.). Herold et.al. claimed lung epithelial cells increase DR5 expression during influenza infection and this resulted in epithelial cell death and severe disease (Herold et al., 2008). However, the antigen used to define epithelial cells, T-1 α , is not specific to epithelial cells, as it is also

highly expressed on endothelial cells. As lung endothelial cells exclusively respond to IFN $\alpha\beta$ signalling, it is possible that type I IFN can induce DR5 expression and subsequent apoptosis of this cell type, which leads to severe influenza induced disease. Since endothelial cells have also been shown to produce proinflammatory cytokines in response to IAV infection, it is possible that this cell type is providing the, as yet unknown, additional signal that induced DR5 upregulation on airway epithelial cells.

We have demonstrated that high frequency of hyper responsive pDCs leads to excessive pulmonary levels of IFN $\alpha\beta$ during X31 influenza infection and consequently epithelial damage-associated mortality of 129 mice. Superficially, it appears that increased susceptibility is due to the quantitative IFN difference seen between 129 and B6 mice. However, as the bone marrow chimera data demonstrates, even with reduced pulmonary IFN $\alpha\beta$ concentrations (as seen in 129 mice reconstituted with IFN $\alpha\beta$ R $^{-/-}$ (129) bone marrow) 129 mice remain susceptible in influenza-induced disease. Our data indicates that this is due to IFN $\alpha\beta$ R dependent upregulation of DR5 on epithelial cells and resultant cell death. Furthermore, it can be argued that due to self-amplification loop of IFN $\alpha\beta$, the excessive levels of IFN $\alpha\beta$ secreted by 129 pDCs are another read out of hyper responsiveness to the IFN $\alpha\beta$ R. Strain specific differences in pDC frequency may be a downstream effect of strain specific differences in responsiveness to IFN $\alpha\beta$. Thus, it may not be a quantitative levels of IFN $\alpha\beta$ that cause 129 pathology but instead a qualitative difference in response to binding of the IFN $\alpha\beta$ R that causes 129 mice to become high responders, resulting in features such as increased IFN $\alpha\beta$ secretion by pDCs or importantly, a lower threshold for DR5 induction. Host specific differences in the IFN $\alpha\beta$ R itself or downstream signalling molecules may

lead to greater responsiveness to the IFN $\alpha\beta$ signal, and possibly may result in an environment where cells are more primed to respond to IFN $\alpha\beta$. We therefore hypothesise that the 129 strain is intrinsically more sensitive to type I IFN signalling. In addition, it must be remembered that the difference between 129 and B6 strains is not limited to an inequality in IFN $\alpha\beta$ responsiveness, undoubtedly other differences between the two strains could contribute to the phenotypes assessed here. Indeed, the 129 strain as a whole appears to be more sensitive to IAV infection and genetic deletion of the IFN $\alpha\beta$ R from 129 mice doesn't bring resistance to IAV induced disease to a comparable level of resistance observed in B6 or IFN $\alpha\beta$ R $^{-/-}$ (B6) mice. Mice from the 129 strain are slower to clear IAV infection than strains tested on the B6 background and trend to have lower levels of IAV specific antibodies produced after IAV infection. Furthermore, assessment of H & E sections of IAV infected lungs revealed that 129 lungs had markedly lower indicative signs of regeneration compared to B6 lungs (data not shown). Host intrinsic factors in the 129 strain, independent of the IFN $\alpha\beta$ driven pathway delineated in this chapter, must contribute to overall susceptibility of this particular inbred mouse strain, however this is beyond the scope of this thesis.

Collectively, we demonstrate that in response to infection by identical influenza virus, which results in the same viral titres detected in the lung throughout the early phase of infection, IFN levels rapidly diverge, depending on the mouse strain background. Host-intrinsic factors are therefore important to determine the magnitude of the IFN response and this response, as we show here through both genetic and cell ablation experiments, can be protective or pathogenic. Induction of a proinflammatory response and epithelial cell death mediated by pDC-derived IFN $\alpha\beta$ may restrict influenza replication; however if

unchecked causes host morbidity and mortality. In the human population, the at-risk group for severe influenza may contain individuals with high frequencies of pDCs or a propensity to strong IFN responses. Thus, this research has important implications for prediction of susceptibility to severe influenza and subsequent treatment of disease induced by this infection.

Chapter 4. Therapeutic potential of IFN α and IFN λ in IAV infection

Work in this Chapter was done in a joint project with Miss Teresa McCabe. Where indicated, experiments were carried out co-operatively. All microarray analysis performed in this chapter was performed out under close supervision by Dr Stefania Crotta.

4.1 Background

IAV causes three to five million cases of severe illness and up to 500,000 deaths world wide, annually (Organization, 2015). IAV is also capable of causing devastating pandemics, as typified by the 1918 ‘Spanish Flu’ outbreak that resulted in an estimated 40 million deaths (Kash et al., 2006). The more recent spread of H1N1, S-OIV resulted in up to 203,000 deaths worldwide, 62%–85% of which were people under the age of 65 years (Simonsen et al., 2013). It is hypothesised that the higher mortality rate in individuals aged less than 65 years elicited by H1N1 S-OIV was due to the antigenic novelty of H1N1 S-OIV compared to circulating IAV strains of the previous five decades (Hancock et al., 2009; Skountzou et al., 2010).

Immunization with inactivated or live vaccines is the best prophylactic option for protection from IAV. However, IAV vaccines must be matched to the current circulating strain, as existing vaccines do not induce broadly neutralizing antibodies and therefore are unable to induce heterotypic and heterosubtypic immunity against the divergent IAV strains (Krammer et al., 2015). Broadly neutralising antibodies to IAV have been isolated in nature. HA stalk-reactive antibodies are able to neutralise multiple HA subtypes by binding to epitopes on the membrane proximal of HA molecule. This region of HA is comparatively well conserved between families of HA subtypes, and therefore, stalk-reactive

antibodies are able to bind to divergent HAs (DiLillo et al., 2014; Ekiert et al., 2011; Tan et al., 2012; Tan et al., 2014). Additionally, antibodies to NA, NP and M2 have also been shown to have broad IAV strain neutralising capacity (Carragher et al., 2008; Zebedee and Lamb, 1988) (Mozdzanowska et al., 1999). However, antibodies which target HA-stem, NP, NA and M2 are immune-subdominant to antibodies reactive to the globular head of HA and therefore are only induced in very low titres in natural infection. Although much effort is being put into developing IAV vaccines that induce heterosubtypic immunity a universal IAV vaccine is still a long way off. Current influenza antivirals include ion channel blockers and NA inhibitors, which act directly on viral proteins (Jefferson et al., 2006). Targeting IAV directly drives the emergence of drug resistant strains due to the high natural mutation rate of IAV. In order to bridge the gap between emerging strains that we do not have vaccines for, without driving pathogen drug resistance, host-directed therapies which stimulate features of the immune response, such as IFNs, should be developed. Induction of pleiotropic cytokines such as IFN $\alpha\beta$ or IFN λ during IAV infection may be less likely to induce IAV mutations. As IAV has already evolved mechanisms to antagonise the induction and action of IFN $\alpha\beta$ and IFN λ and given the multiplicity of antiviral effectors that are induced by IFNs, addition of more of these cytokines to an infected system may serve to circumvent virus mediated block of IFNs while also making it difficult for IAV to evolve mutants to escape such a multifaceted antiviral response.

As already discussed (Chapters 1 and 3), Type I IFNs are a family of antiviral cytokines. All type I IFN subtypes act through a common, ubiquitously expressed, heterodimeric receptor (IFN $\alpha\beta$ R) to induce the transcription of a diverse set of genes known as ISGs (Randall and Goodbourn, 2008). More recently

discovered, type III IFNs (IFN λ 1, 2, 3 and 4) are also induced during viral infection and utilise the same JAK/STAT signalling pathway as type I IFN to activate the same ISGs (Kotenko et al., 2003; Sheppard et al., 2003). IFN λ s bind to an independent receptor complex consisting of IL-10R2 and IFNLR1. Unlike the ubiquitously expressed IFN $\alpha\beta$ R, IFN λ R is restricted primarily to mucosal surfaces such as the lung epithelial layer (Mordstein et al., 2008; Sheppard et al., 2003; Sommereyns et al., 2008). Infection of IFN $\alpha\beta$ R/IFN λ R double deficient mice with a panel of respiratory pathogens including IAV, revealed that the lungs of these mice were highly permissive to viral replication. IFN $\alpha\beta$ R $^{-/-}$ /IFN λ R $^{-/-}$ mice had significantly higher titres of IAV, IBV, RSV, HMPV and SARS coronavirus compared not only to Wt mice, but also mice deficient for only IFN $\alpha\beta$ R or IFN λ R. Increased virus load in IFN $\alpha\beta$ R $^{-/-}$ /IFN λ R $^{-/-}$ mice correlated to higher disease burden and host mortality (Mordstein et al., 2008). A further study confirmed that IFN $\alpha\beta$ R and IFN λ R signalling is entirely redundant in AECs and only genetic ablation of both IFN $\alpha\beta$ R and IFN λ R in AECs in vivo resulted in high IAV loads and host morbidity and mortality, in spite of a Wt immune system (Crotta et al., 2013). Induction of antiviral ISGs in AECs is therefore of critical importance to control of influenza. Of particular relevance to IAV, the ISG: interferon-inducible transmembrane protein 3 (IFITM3) has been demonstrated to assist in restriction of virus replication in mice and in some human populations and mutation in the *ifitm3* gene leads to increased IAV induced disease burden (Everitt et al., 2012; Zhang et al., 2013). Additionally, the appropriately named orthomyxovirus resistance gene family (Mx family) also exhibits potent IAV restriction capabilities. Murine Mx1 accumulates in the nucleus of infected cells and interferes with primary IAV transcription by truncating transcripts encoding IAV polymerase proteins. In contrast, the human homologue,

MxA has been shown in vitro to restrict IAV replication by interfering with viral protein transport, synthesis or translocation (Pavlovic et al., 1992).

Induction of antiviral genes by IFNs has been extensively demonstrated to restrict IAV replication in vitro. Pretreatment of mice which can express a functional Mx1 protein with IFN α has been shown to markedly impede 1918 H1N1 IAV replication in the lung as well as curbing cytokine and chemokine gene expression, compared to non IFN α treated IAV infected controls. Lower viral load and inflammatory response correlated with decreased necrotizing bronchiolitis and associated peribronchiolar lymphocytic inflammation (Cilloniz et al.). Pretreatment with IFN α also protected functional Mx1 mice from high virus load in the lung and mortality in H5N1 infection (Tumpey et al.). Treatment of ferrets with IFN α prior to infection with a seasonal IAV strain did assist in virus control and resulted in lower clinical scores compared with mock-treated controls; continuing IFN α treatment to days 1 and 2 postinfection increased the positive treatment outcome. However, protection was not conferred when ferrets were challenged with avian IAV (Kugel et al.). Finally, pretreatment with IFN α protected guinea pigs from both seasonal and highly pathogenic strains of IAV (Van Hoeven et al.).

As the human population expands, the interface between the animal reservoir of IAV and the human population grows. Increased contact increases the likelihood of a novel IAV strain to cross the species barrier. Coupled with the difficulty of developing an IAV vaccine that will induce broad protection, the time lag between vaccination and host protection, and IAV's exceptional ability to escape host adaptive immunity through antigenic shift and drift, development of a treatment that will stimulate protective aspects of the host immune response to IAV is highly desirable. In this context, type I IFN has been periodically discussed

as a possible treatment for IAV during infection (Finter et al., 1991; McKinlay, 2001; Wang et al., 2014). Yet, results presented in Chapter 3 strongly argue that there are limits to this. We demonstrated that prolonged or exaggerated type I IFN signalling during IAV infection can exacerbate, not ameliorate disease (Davidson et al.). However, it is important to note that all studies performed in Chapter 3 were done in mouse strains whose Mx1 gene codes for a non-functional product. Given the potent IAV restriction abilities of functional Mx1 protein it is therefore possible that the pathogenic potential of IFN $\alpha\beta$ we see in 129 mice may only be observable in an Mx1 null background. This is particularly important as the human homologue of Mx1 (MxA) has also been demonstrated to restrict IAV in vitro (Pavlovic et al.). To clarify this issue and assess whether or not IFN $\alpha\beta$ has therapeutic potential for IAV, we decided to assess the effect of administration of exogenous IFN $\alpha 4$ during IAV infection. However, being aware of the potent immunomodulatory effects of IFN $\alpha\beta$ signalling we also decided to include IFN $\lambda 2$ treatment in our study. IFN λR is largely restricted to mucosal surfaces in the lung and as such IFN $\alpha 4$ and IFN $\lambda 2$ treatment during IAV infection may stimulate different cell types and therefore may lead to divergent outcomes.

4.2 Hypothesis and Aims

I aimed to determine whether or not either (or both) IFN α 4 and IFN λ 2 are viable treatment options for IAV-induced disease. We define a viable treatment option as one that protects host tissue from damage and lowers overall disease burden experienced by the host.

I hypothesised:

- Both IFN α 4 and IFN λ 2 treatment during IAV infection will inhibit viral replication.
- Due to different tissue distribution of the IFN $\alpha\beta$ and IFN λ receptors, IFN α 4 will stimulate the immune system leading to pathology, while IFN λ 2 will not.

4.3 Results

4.3.1 Pretreatment with IFN α 4 or IFN λ 2 blocks IAV infectivity

To determine comparable doses of antiviral effect in infected cells for IFN α 4 and IFN λ 2 we assessed induction of the antiviral ISGs: Mx1, Rsad2, Oasl2 and ifi203 in B6 derived AEC cultures. IFN α 4 and IFN λ 2 were titrated on AEC cultures from above ISG induction saturation over 9 points down to non-induction. These results were used to generate dose response curves for IFN α 4 and IFN λ 2 treatment, for each ISG assessed. For each response curve, EC₅₀ values were obtained and used to generate a conversion ratio for equipotency between IFN α 4 and IFN λ 2 (Data from this analysis will not be presented here as they are to be included in the thesis of the collaborating student, Teresa McCabe). Using this conversion factor we were able to treat mice and various cell types with doses of IFN α 4 and IFN λ 2 that we defined to induce equivalent ISG induction throughout our study.

As has been previously shown in other studies (Cilloniz et al., 2012; Mordstein et al., 2008; Tumpey et al., 2007), intranasal IFN treatment of mice prior to infection with IAV protected Mx1 functional mice (B6.A2G-Mx1) from IAV strain PR8 induced morbidity and mortality. This protection correlated with undetectable viral loads at 4 dpi in IFN treated groups, as compared to control group mice (Veh Ctrl), which did exhibit higher viral loads, weight loss and 50% mortality (Figure 27).

Figure 27

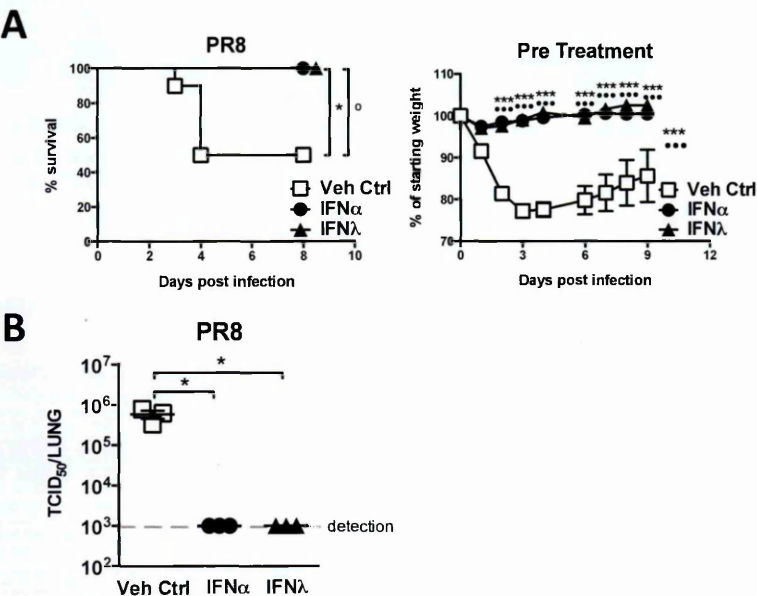


Figure 27: Pretreatment with IFNα4 or IFNλ2 ablates disease burden and facilitates virus control. (A) B6.A2G-Mx1 mice were pretreated with equivalent doses of IFNα4 (circles) or IFNλ2 (triangles), or Veh Ctrl (open squares) 24hrs prior to infection with PR8; weight loss and survival was assessed throughout infection and (B) viral load assessed at 4dpi. Significance assessed by Log-rank (Mantel-Cox) test (survival), 2-way ANOVA Bonferroni post tests (weight loss) and Unpaired t tests (viral load). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, where * indicates IFNα4:Veh Ctrl and ° indicates IFNλ2:Veh Ctrl, IFNα4:IFNλ2; was not significant. Symbols on the right of graphs indicate statistical significance of the whole curve and symbols above individual points indicate significance as assessed by Bonferroni post test. Graphs show mean \pm SEM and unless otherwise stated are representative of 2 independent experiments where $n=3-6$.

4.3.2 Overlapping and nonredundant effects of IFN α 4 and IFN λ 2

To understand why pretreatment was so effective at protecting mice from IAV induced morbidity and mortality, we assessed the response of specific cell types present in the lung during IAV infection to IFN stimulation. As they are the primary infection targets of IAV, we first evaluated the response to IFN treatment in AEC cultures generated from B6 mice. Both IFN α 4 and IFN λ 2 induced expression of ISGs in AECs, however neither IFN treatment induced cytokine secretion from these cultures (Figure 28A, B). In contrast, macrophages, pDCs and cDCs derived from B6 bone marrow only responded to IFN α 4 stimulation, IFN λ 2 treatment of these cell types did not elicit expression of tested ISGs (Figure 28C). Furthermore, as assessed at 24hrs post stimulation macrophages, pDCs and cDCs all secreted proinflammatory cytokines in response to IFN α 4 treatment and again, did not respond to IFN λ 2 (Figure 28D). Similarly, ex vivo stimulation of whole splenocyte cultures with IFN α 4 and not IFN λ 2 led to the upregulation of activation markers; Sca-1 and CD69, on T cells, B cells and NK cells (Figure 29). Collectively these results indicate that AEC upregulation of antiviral ISGs upon stimulation with either IFN α 4 or IFN λ 2 may be sufficient to inhibit IAV establishing an infection in vivo. Induction of proinflammatory cytokines by IFN α 4 treatment in immune cells appears unnecessary for pretreatment mediated protection.

Yet, culture of specific cell types alone does not allow for observation of cross talk between cell types. As such, we assessed the global transcriptional response in whole lungs treated with IFN α 4, IFN λ 2 or Veh Ctrl. Mice were treated with IFN α 4, IFN λ 2 or Veh Ctrl, and 18hrs later whole lungs were collected and processed for microarray analysis. Samples were normalised to the average of the Veh Ctrl group and filtered for a fold change of 1.5, giving 553 genes differently

Figure 28

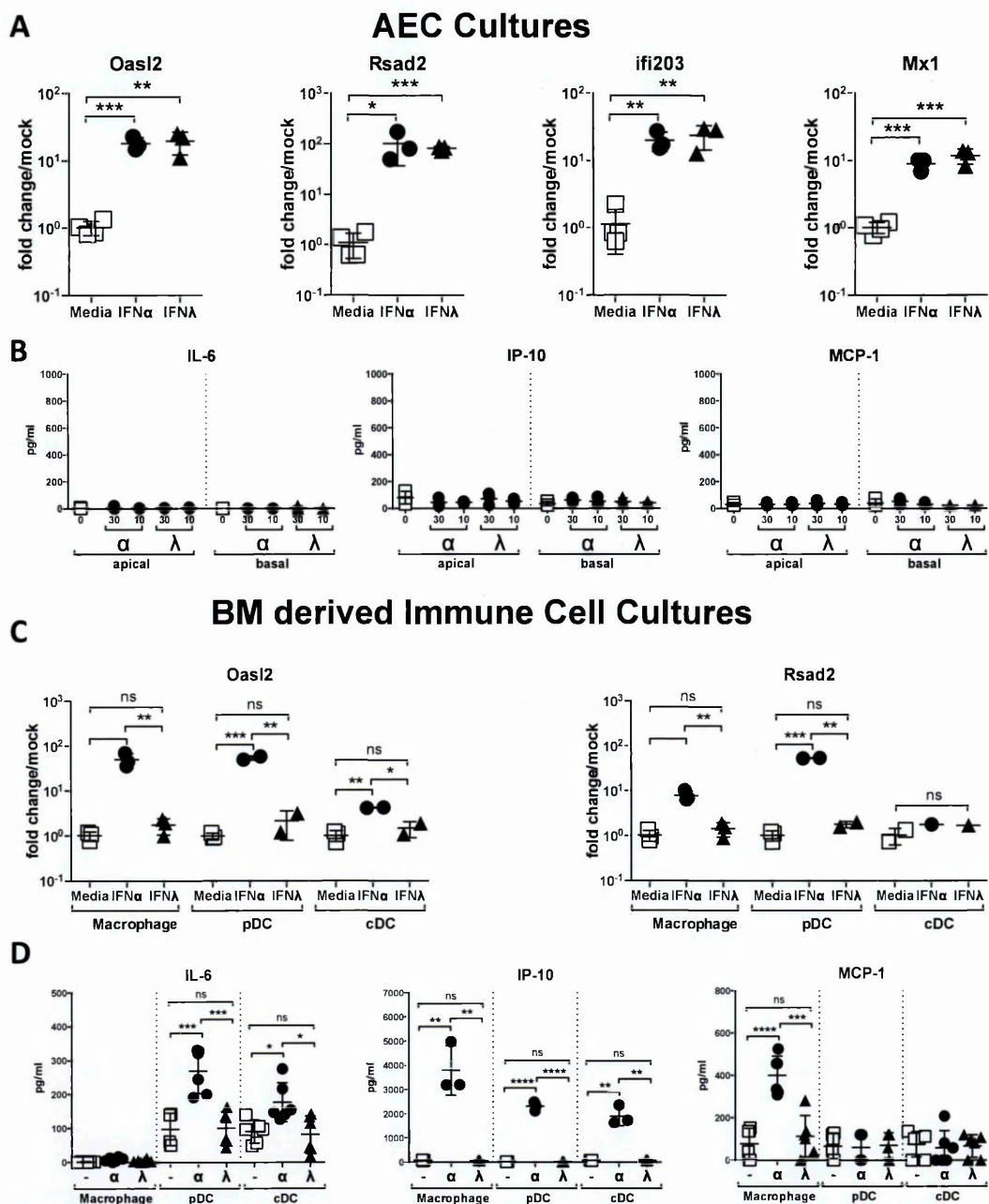


Figure 28: AECs upregulate ISGs in response to IFN α 4 or IFN λ 2 stimulation while only IFN α 4 stimulation elicits ISG expression and cytokine secretion from BM derived immune cells. ISG expression in (A) B6 derived AEC cultures or (C) B6 BM derived Macrophage, pDC and cDC cultures stimulated with IFN α 4 or IFN λ 2 for 4 hrs. IL-6, IP-10 and MCP-1 concentrations were measured by multiplex in (B) AEC culture supernatants collected from both apical and basal sides of culture transwells and (D) Macrophage, pDC and cDC culture supernatants at 24hrs post stimulation with IFN α 4 or IFN λ . Significance assessed by Unpaired t tests where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of 2 independent experiments where $n=3-6$. *AEC Samples collects and processed with Miss Teresa McCabe.*

Figure 29

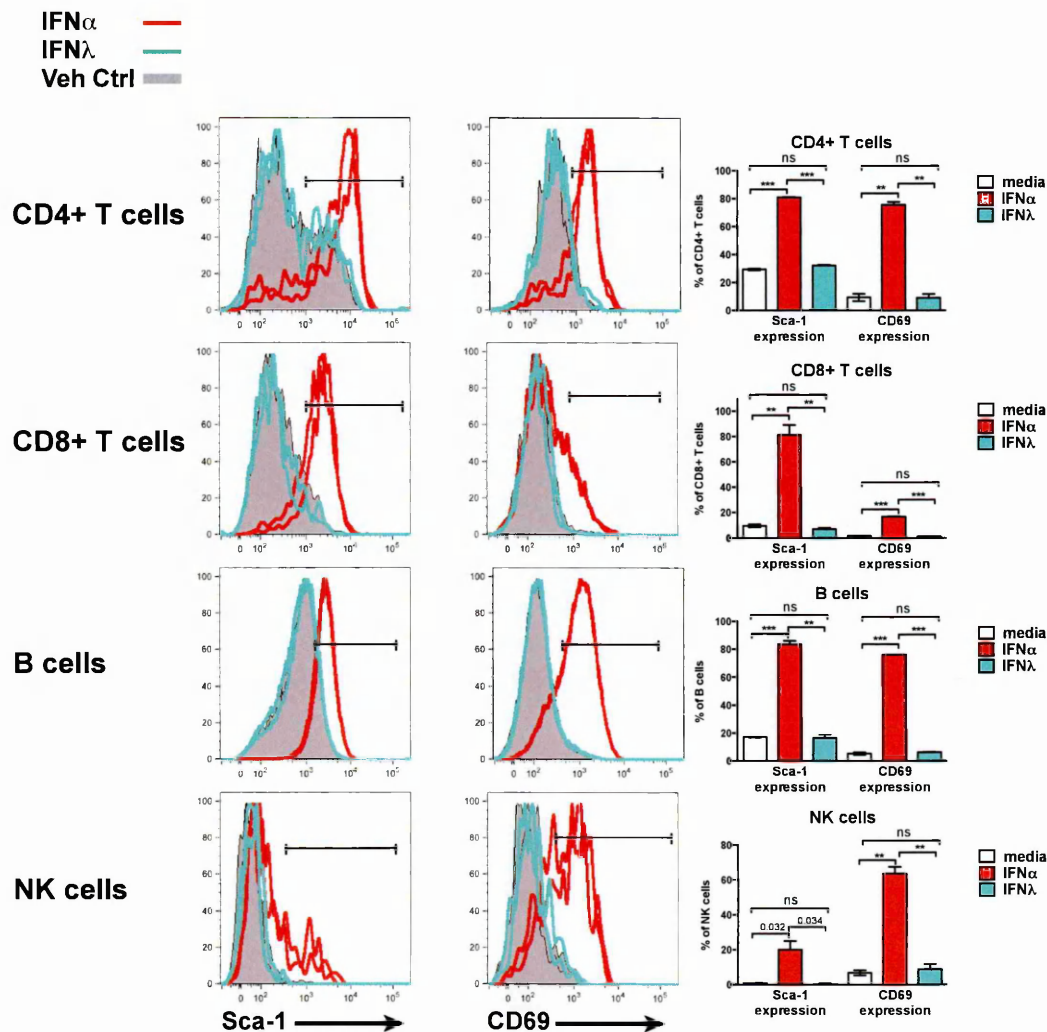


Figure 29: Splenic T, B and NK cells upregulate Sca-1 and CD69 upon IFN α 4, but not IFN λ 2 stimulation. Whole splenocytes isolated from B6 mice were stimulated with IFN α 4 (red line) or IFN λ 2 (blue line) and along with Veh Ctrl (filled grey) expression of Sca-1 and CD69 at 24hrs assessed on CD4⁺ T cells, CD8⁺ T cells, B cells and NK cells by flow cytometry. Significance assessed by Unpaired t tests where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of 2 independent experiments where $n=3$.

regulated between treatments. Genes upregulated by IFN α 4 or IFN λ 2 treatment were subjected to K means clustering (Figure 30). Of the 6 clusters we defined, 5 contained genes modulated by both IFN α 4 and IFN λ 2 to varying magnitudes (Figure 30A). Overall, lungs treated with IFN α 4 exhibited gene upregulation of greater magnitude than IFN λ 2 treated lungs, however this is likely a reflection of the difference in cell types that could potentially respond to each IFN treatment. IFN λ R expression is restricted to AECs in the lungs while IFN α β R is ubiquitously expressed (Mordstein et al., 2008; Sommereyns et al., 2008). In contrast to the other clusters, cluster 6 revealed genes almost exclusively induced by IFN α 4 (Figure 30B). Ingenuity pathway analysis (IPA) of the combined clusters 1-5 revealed genes in this group were strongly related to IFN signalling pathways and communication between immune cells, while cluster 6 genes were annotated as genes involved in cellular recruitment pathways and inflammation, and interestingly, 'Role of hypercytokinemia/ hyperchemokineemia in the pathogenesis of influenza' (Figure 30C, D and Gene lists in Appendix Tables 2 and 3).

IPA analysis of genes specifically regulated by IFN α 4 treatment and the in vitro response of immune cells we tested strongly suggested that IFN α 4 but not IFN λ 2 treatment would induce secretion of proinflammatory cytokines in the lung. To confirm this we assessed levels of example proinflammatory cytokines: IL-6, IP-10 and MCP-1 in BAL fluid taken at 10, 18 and 48hrs post IFN treatment. Detection of significant IL-6, IP-10 and MCP-1 concentrations demonstrated that indeed, proinflammatory cytokine secretion was induced in lungs treated with IFN α 4, yet not by IFN λ 2 or Veh Ctrl treatment (Figure 31). Taken together, our results indicate that both IFN α 4 and IFN λ 2 induce antiviral gene expression in whole lungs and this induction prior to IAV infection protects the host from severe

Figure 30

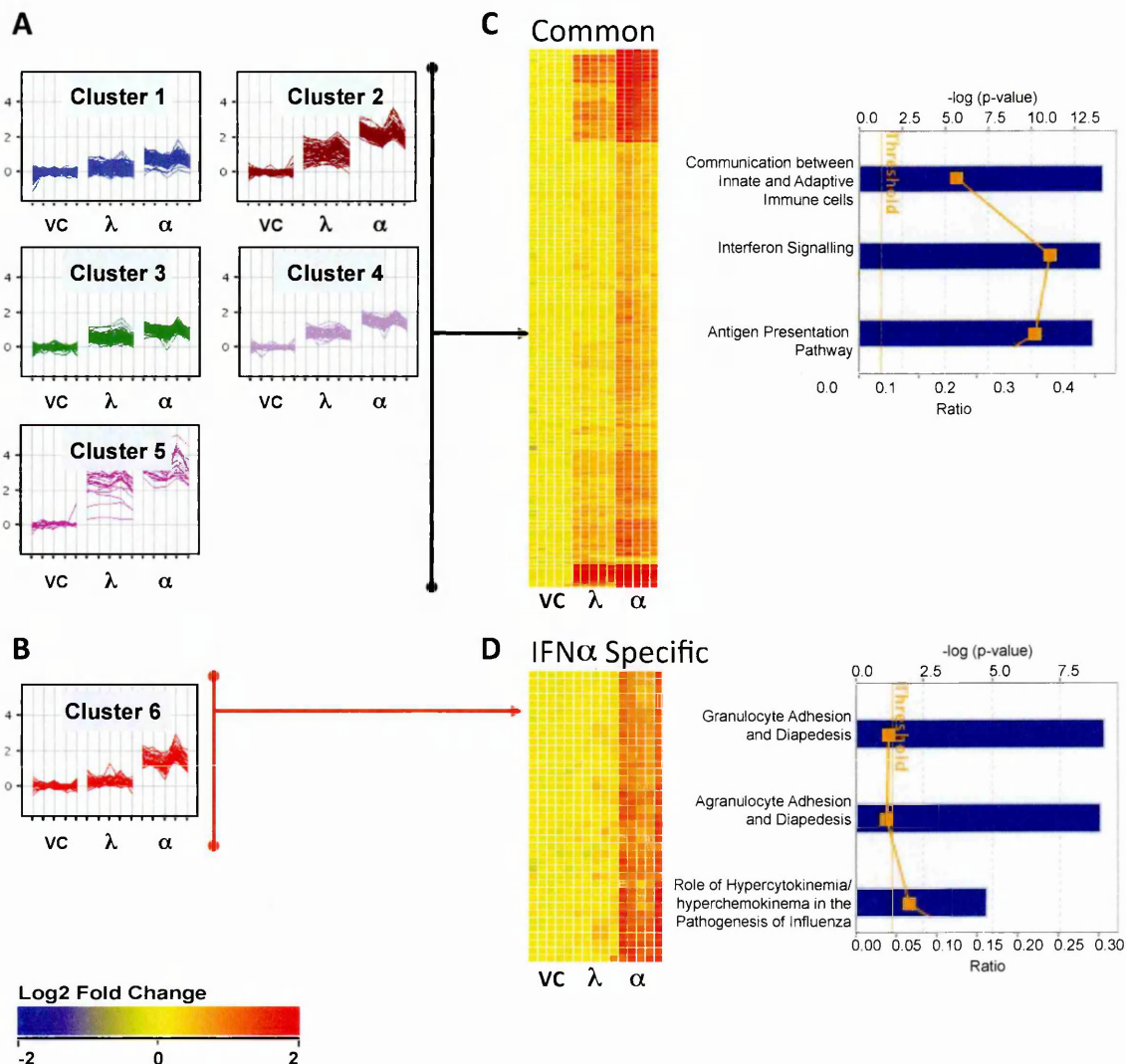


Figure 30: Inflammatory-related gene clusters are specifically induced by IFNα4, not by IFNλ2 treatment in whole lung samples. B6 mice were treated with IFNα4, IFNλ2 or Veh Ctrl and whole lungs were taken at 18hrs post treatment for global analysis by Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Samples were normalised to the median of the vehicle control group and filtered for a fold change of 1.5, yielding 553 genes differently regulated between treatments (One way ANOVA, $P < 0.01$, Benjamini-Hochberg multiple test correction), of which 429 genes are upregulated. K means clustering of upregulated genes revealed two patterns of expression: (A) genes upregulated by both IFNα4 and IFNλ2 treatment (common) and (B) genes primarily induced by IFNα4. (C, D) Clusters 1-5 were grouped together and Common and IFNα specific groups were analysed by Ingenuity Pathway Analysis (IPA). *Samples collect and processed with Miss Teresa McCabe and analysis performed under supervision of Dr. Stefania Crotta.*

Figure 31

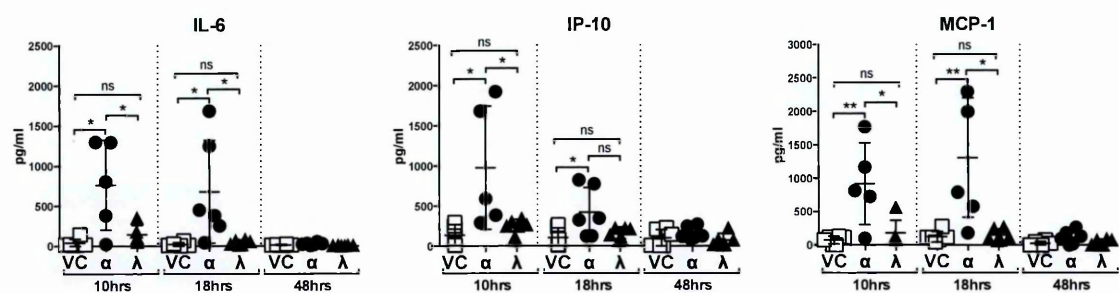


Figure 31: IFN α 4, but not IFN λ 2 treatment induces cytokine secretion in the pulmonary environment. BAL samples taken from B6 mice treated with IFN α 4, IFN λ 2 or Veh Ctrl at specified time points. Significance assessed by Unpaired t tests where *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are representative of 2 independent experiments where n=3-6.

disease burden. Stimulation of immune cells and induction of proinflammatory cytokine secretion is a function specific to IFN α 4 treatment. As was demonstrated in Chapter 3, IFN α β induced inflammation must be carefully controlled as too little (as seen in IFN α β R $^{-/-}$ (B6) mice) or too much (Wt 129 mice) can increase host morbidity. IFN α 4 specific induction of genes associated with inflammation therefore has the potential to be protective or detrimental.

4.3.3 IFN α 4 and IFN λ 2 treatment during IAV infection results in divergent disease outcomes

Induction of antiviral ISGs in the lung prior to infection markedly impedes IAV infectivity. However, it is not a realistic option to 'pretreat' an entire human population. Therefore, we next assessed the effectiveness of IFN α 4 and IFN λ 2 treatments at ameliorating disease during IAV infection. B6.A2G-Mx1 mice were infected with PR8 and treated intranasally with IFN α 4, IFN λ 2 or vehicle control at days 2, 4 and 5 post infection (Figure 32A). IFN λ 2 treated mice were protected against severe IAV-induced disease, exhibiting significantly lower mortality and morbidity compared to the Veh Ctrl group. In striking contrast, IFN α 4 treated mice were not protected, instead IFN α 4 treatment exacerbated disease. We observed significantly higher morbidity and mortality in IFN α 4 treated mice than that observed in the control or IFN λ 2 groups (Figure 32B). Interestingly, while disease outcome was entirely divergent between the IFN treatment groups, viral load in the lung was comparable. IFN α 4 and IFN λ 2 treated lungs exhibited significantly lower viral loads compared to lungs treated with Veh Ctrl (Figure 32C).

Given that control of viral replication was comparable between IFN α 4 and IFN λ 2 we hypothesised that similar to results detailed in Chapter 3, treatment with

Figure 32

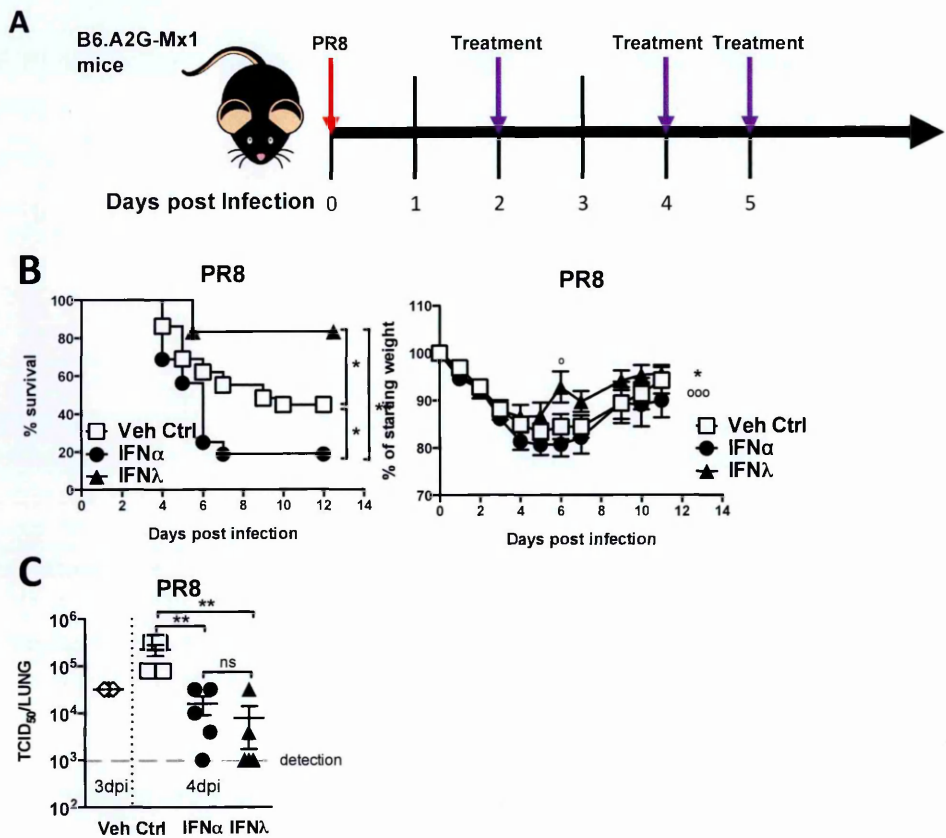


Figure 32: IFN α 4 treatment exacerbates PR8 induced disease while IFN λ 2 treatment protects. (A) Treatment regime: B6.A2G-Mx1 mice were infected with PR8 (red arrow) and treated with equivalent doses of IFN α 4 or IFN λ 2 or Veh Ctrl at days 2, 4 and 5 post infection (purple arrows); (B) survival and weight loss was monitored (data is pooled from 4 independent experiments where n=6-9 per experiment) and (C) viral load assessed at 4dpi. Significance assessed by Log-rank (Mantel-Cox) test (survival), 2-way ANOVA (weight loss) and Unpaired t tests (viral load). *, P < 0.05; **, P < 0.01; ***, P < 0.001, where * indicates IFN α 4:Veh Ctrl and ° indicates IFN α 4:IFN λ 2; IFN λ 2:Veh Ctrl was not significant. Symbols on the right of graphs indicate statistical significance of the whole curve.

Figure 33

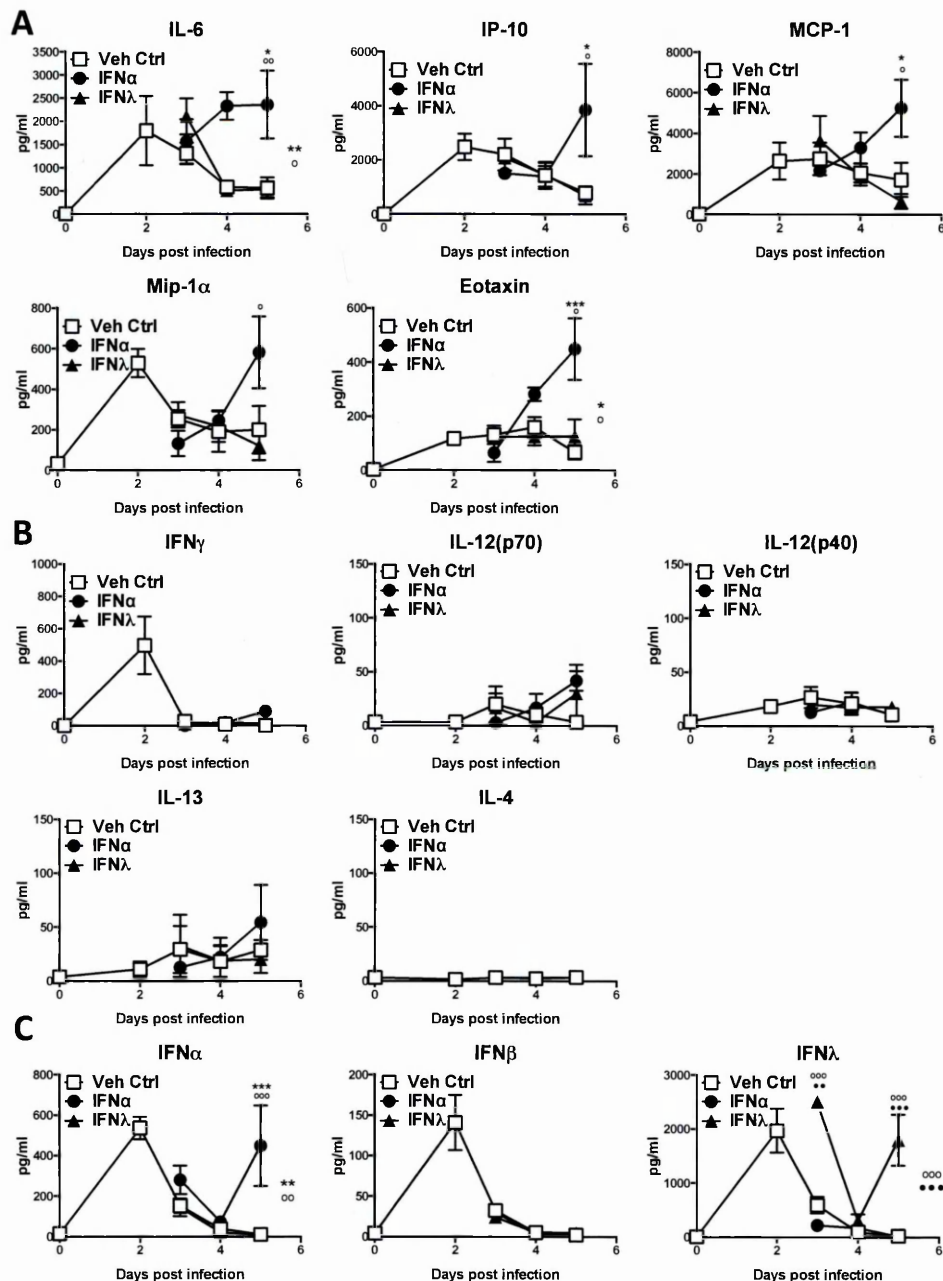


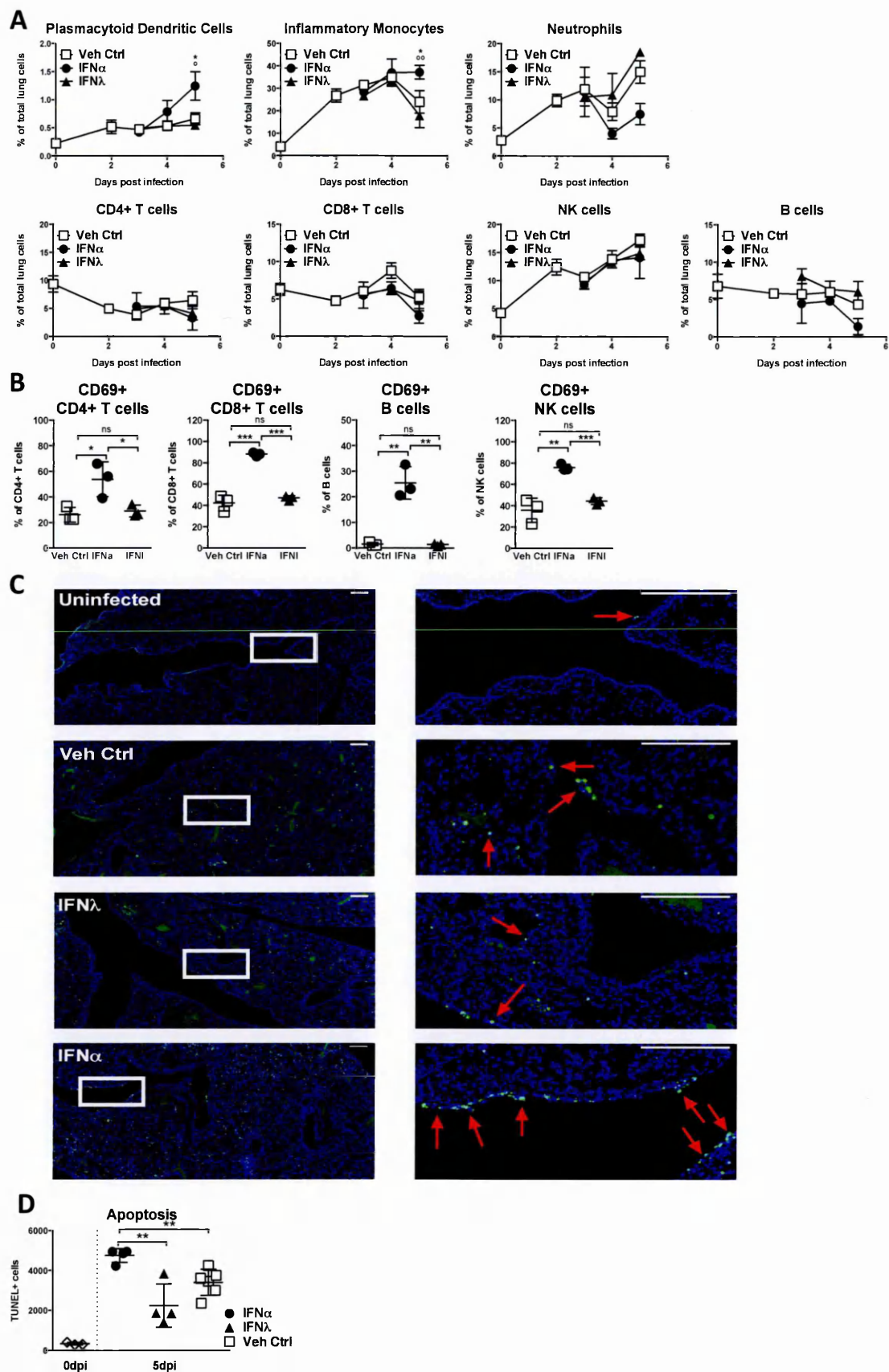
Figure 33: IFN α 4 treatment causes with increased proinflammatory cytokine secretion during IAV infection. B6.A2G-Mx1 mice were infected with PR8 and treated with IFN α 4 (circles), IFN λ 2 (triangles) or Veh Ctrl (open squares) as per Figure 32 A. (A, B) Concentrations of stated proinflammatory cytokines in BAL fluid was measured by multiplex. (C) Concentrations of IFN α , β and λ were assessed by ELISA. Significance assessed by 2-way ANOVA with Bonferroni post tests (where * denotes IFN α 4:Veh Ctrl and ° indicates IFN α 4:IFN λ 2. IFN λ 2:Veh Ctrl was not significant). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) while those above indicate significance of individual time points (Bonferroni post test). *, P < 0.05; **, P < 0.01, ***, P < 0.001 graphs show mean \pm SEM and are representative of 2 independent experiments where n=3.

exogenous IFN α 4 during IAV infection was over stimulating the immune system leading to immunopathology. We therefore assessed pulmonary levels of proinflammatory cytokines, cellular recruitment and epithelial cell death during infection. IFN α 4 treatment augmented concentrations of IL-6, IP-10, MCP-1, Eotaxin and Mip-1 α in BAL from infected mice. In contrast, proinflammatory cytokine levels in Veh Ctrl and IFN λ 2 treated mice were comparable throughout PR8 infection (Figure 33A). IFN treatment did not alter concentrations canonical Th1 (IFN γ , IL-12(p40) and IL-12(p70)) or Th2 (IL-4 and IL-13) cytokines in IAV infected lungs (Figure 33B). IFN α 4 treated mice had higher levels of IFN α , as assessed by ELISA at 5dpi, yet not IFN β and IFN λ (Figure 33C). This may be due to the IFN α β driven positive feedback loop or, more likely, detection of exogenous IFN α 4 administered on 4dpi. Similarly, pulmonary levels of IFN λ as assessed by ELISA were elevated in IFN λ 2 treated mice at days 3 and 5 post infection.

Recruitment of CD4⁺ T cells, CD8⁺ T cells and NK cells was comparable between all treatment groups, while recruitment of Neutrophils and B cells was somewhat blunted in the IFN α 4 treatment group, however this was not

Figure 34: IFN α 4 but not IFN λ 2 treatment enhances activation and recruitment of immune cell subsets and AEC death in PR8 infection. (A-C) B6.A2G-Mx1 mice were infected with PR8 and treated with IFN α 4 (circles), IFN λ 2 (triangles) or Veh Ctrl (open squares) as per Figure 32A. (A) pDC, IMc, Neutrophil, CD4⁺ T cell, CD8⁺ T cell, NK cell and B cell recruitment was assessed by flow cytometry. (B) Activation of T, NK and B cells was also assessed by CD69 expression at 5dpi. (C) Lung sections from control and infected mice treated as indicated, were stained by TUNEL for apoptotic cells at 6dpi. Red arrowheads indicate TUNEL signal. Scale bar, 200 μ M. (D) Quantification of TUNEL⁺ cells in whole lung slides by Icy Spot Detector (ICY-R3M2Y2). Significance assessed by 2-way ANOVA with Bonferroni post tests (where * denotes IFN α 4:Veh Ctrl and ° indicates IFN α 4:IFN λ 2. IFN λ 2:Veh Ctrl was not significant) (time course) or Student's t test (CD69 expression and TUNEL quantification). Symbols on the right of graphs indicate statistical significance of the whole curve (2 way ANOVA) and those above indicate significance of individual time points (Bonferroni post test). *, P < 0.05; **, P < 0.01, ***, P < 0.001, graphs show mean \pm SEM and are representative of 2 independent experiments where n=3.

Figure 34



significant (Figure 34A). T cells, B cells and NK cells all had high expression of the activation marker CD69 at 5dpi in IFN α 4 treated mice, while CD69 expression was significantly lower on these cell types in control and IFN λ 2 treated mice (Figure 34B). Interestingly, higher frequencies of pDCs and IMcs were observed in IFN α 4 treated lungs at 5dpi (Figure 34A). Increased numbers of IMcs associated with higher numbers of apoptotic airway epithelial cells, as assessed by TUNEL staining of lung sections. In contrast, treatment with IFN λ 2 resulted in a lower frequency of apoptosis in airway epithelial cells, compared to both IFN α 4 and Veh Ctrl treatment (Figure 34C). Thus, IFN λ 2 treatment controls viral replication efficiently and without the extensive inflammation and apoptosis of airway epithelial cells associated with IFN α 4 treatment. It appears, in the context of a functional immune response, further stimulation by IFN α 4 leads to over activation of the immune response and tissue damage rather than protection.

4.3.4 IAV tissue tropism and IFNLR expression overlap

Crotta et al. demonstrated that IFN $\alpha\beta$ and IFN λ induce identical ISG suites (Crotta et al.). We therefore must ask why treating with IFN α 4 and IFN λ 2 leads to such divergent disease outcomes. Productive IAV replication is restricted to AECs, which we confirmed by intracellular staining for PR8 nucleoprotein (NP) in hematopoietic (CD45⁺) and non-hematopoietic (CD45⁻) pulmonary cells (Figure 35A). Auspiciously, IFNLR1 expression is also restricted to non-hematopoietic cells of the lung, while, as implied by the results presented in this chapter, IFNAR1 is ubiquitously expressed (Figure 35B). Thus, due to its restricted receptor distribution that matches cell type infectivity of IAV IFN λ 2 treatment delivers IFN

Figure 35

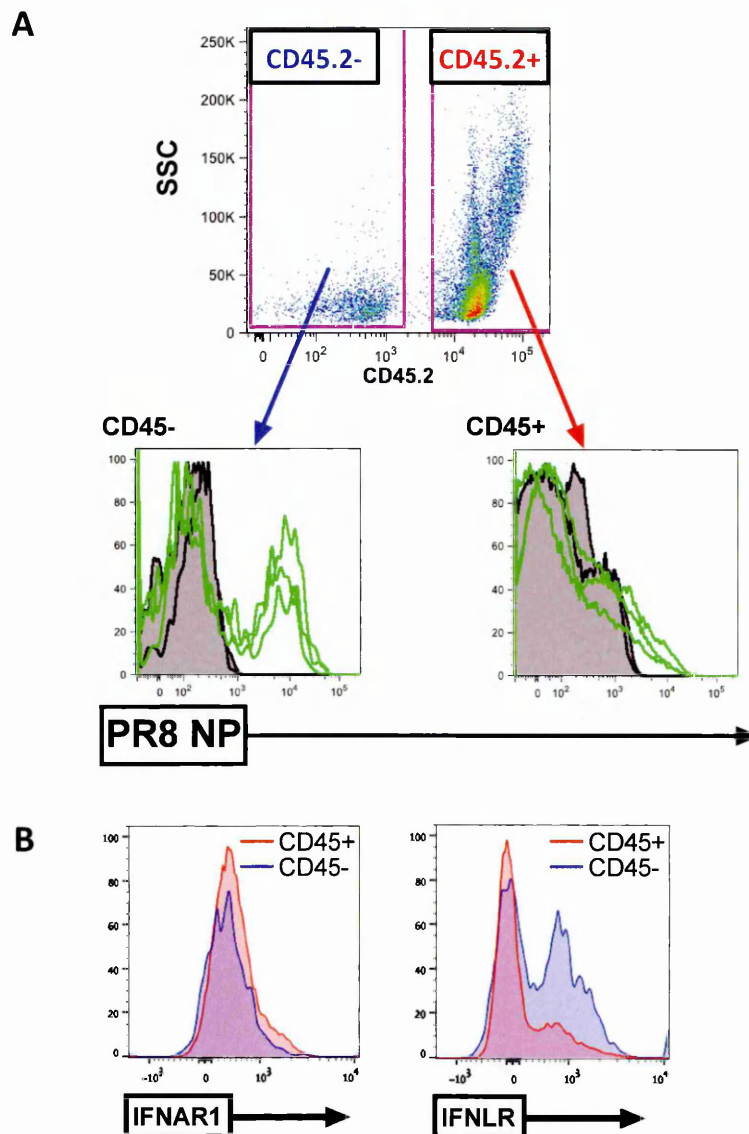


Figure 35: IAV replication and IFNLR1 expression are restricted to CD45⁻ cells in the lung. (A) Flow cytometric analysis of pulmonary cells for PR8 nucleoprotein (NP) was performed on B6 mice at 3dpi. Cells were gated into hematopoietic (CD45⁺) and non-hematopoietic (CD45⁻) populations then visualized by histograms (green) against Veh Ctrl treated lungs (grey). (B) Hematopoietic (CD45⁺, red) and non-hematopoietic (CD45⁻, blue) populations from untreated mouse lungs were assayed for expression of IFNAR1 and IFNLR1. Data is representative of 2 independent experiments where n=2-3.

signalling where required without needlessly stimulating an already activated immune response.

4.4 Discussion

An ideal pan-IAV treatment, designed to be given to an immunocompetent population, should stimulate induction of antiviral genes in AECs, without driving immunopathology. Both IFN α 4 and IFN λ 2 treatments effectively controlled IAV replication, yet only the IFN λ 2 treated group was protected against severe IAV disease. Unlike IFN α 4 treatment, which drove disease features associated with severe IAV, administration IFN λ 2 during IAV infection did not increase proinflammatory cytokine concentrations, recruitment of innate inflammatory cells or frequency of apoptotic AECs in the lung. Further investigation revealed that IFN λ R expression in the lung was restricted to nonhematopoietic cells, which allowed IFN λ 2 treatment to target cells at risk of IAV infection to induce ISG upregulation while not over exciting the immune response. Collectively, this data suggests IFN λ is a strong candidate for a nonstrain specific IAV treatment in humans.

We conclude from the IFN mediated induction of ISGs in AECs (Figure 28A) and the decreased presence of IAV infectious particles in lungs of IFN treated mice at 4dpi, in both pre and post treatment regimes (Figure 27 and 32), that both IFN α 4 and IFN λ 2 effectively inhibit IAV replication. In the case of the B6 background, both IFN $\alpha\beta$ and IFN λ concentrations in the lung post IAV infection are low. This is likely due to not only the mouse strain background, which we demonstrated in Chapter 3 to be a low IFN responder but also due to IAV itself. The NS1 protein of IAV antagonises induction of IFNs by interfering with upstream pathways such as inhibition of RIG-I ubiquitination or activation of IRF-3 (Gack et al., 2007; Hale et al., 2008). Exogenous IFN treatment therefore may control IAV spread through the lung by potentiating ISG expression in uninfected cells or

bypassing the block in IFN induction due to IAV NS1 action (Ehrhardt et al.). Additionally, while control of IAV replication itself to protect against viremia is important, IFN λ 2 treatment may also ameliorate severe disease by minimising inflammation induced by continued virus presence in the pulmonary environment. Prolonged virus occupancy, even at low levels, can perpetuate immune signalling and contribute to tissue damage (Bhattacharya et al., 2014; Napoli et al., 1996). MCP-1 and Mip-1 α as well as IMc recruitment trend to be lower in IFN λ 2 treated mice compared to Veh Ctrl group, and this correlates to a significantly lower frequency of apoptotic AECs, as assessed by TUNEL staining. However to confirm this hypothesis, assessment of these inflammatory parameters should be performed at later time points.

As demonstrated by the results presented in this chapter, virus control is not the only factor that determines host mortality or survival. Generally, severe IAV disease in humans is characterised by cytokine storm and pulmonary tissue destruction that can be virus- or immune-mediated (Beigel et al., 2005; de Jong et al., 2006; Louie et al., 2009; Peiris et al., 2004). Similar to results presented in Chapter 3 with IAV infected 129 mice, exogenous IFN α 4 treatment is upstream of secretion of proinflammatory cytokines, as IFN α 4 treated mice had markedly augmented pulmonary levels of IL-6, IP-10, MCP-1 etc. Enhancement of proinflammatory secretion during IAV infection by IFN α 4 on lung resident cells such as macrophages drives recruitment of pDCs and IMcs to the lung. Concomitantly, these cell types have been demonstrated to secrete proinflammatory cytokines and chemokines such as MCP-1 and IFN α β themselves, which will amplify the immune response by induction of more inflammatory mediators and inflammatory cell recruitment, continued inflammation leading to

AEC apoptosis and ultimately, host pathology. However, it should be noted that this amplification only occurs in the context of IAV infection. Treatment with IFN α 4 of uninfected mice did result in proinflammatory cytokine secretion at 10 and 18hrs post infection yet these cytokines were no longer detected at 48hrs post treatment and as demonstrated in Figure 13 of Chapter 3, repeated treatment with IFN α 4 over a short time period does not induce morbidity or mortality in the host. Moreover, IFN α 4 induced inflammation was associated with protection in the context of pretreatment. Thus, our results demonstrate that IFN α 4 drives immunopathology when added to an immune system that is already responding to a replicating pathogen, however IFN α 4 treatment alone is insufficient to bring about immunopathology in this experimental context.

Importantly, IFN α 4 induced inflammation is designed to be protective, and recruitment of immune cells and apoptosis of potentially infected cells facilitate viral clearance. It is only when over activated, by addition of exogenous IFN α 4 to the system, that tissue damage occurs, leading to serious consequences for the host. As shown in Figure 30, by clustering the response to IFN into genes specifically induced only by IFN α 4 and genes commonly induced by both IFN α 4 and IFN λ 2, we identify a set of genes, specific to IFN α 4, that drive pathogenicity in IAV infection. These genes are primarily proinflammatory cytokines and as observed in human studies high disease burden associates with hypercytokinemia (Arankalle et al., 2010; Beigel et al., 2005; de Jong et al., 2006; Hayden et al., 1998; Peiris et al., 2004), indeed this group of genes scored highly for 'Role of hypercytokinemia/hyperchemokinema in the pathogenesis of influenza' in IPA, a reasonable conclusion we can make from this data is that immune stimulation by IFN α 4 during IAV infection can be harmful to the host. Restricted IFNLR expression

allows IFN λ 2 to target cells at risk of IAV infection with minimal perturbation of immune cell recruitment and cytokine response. If taken together with the results from Chapter 3, it is conceivable that in addition to treating IAV infection in humans with IFN λ it may also be advantageous to block type I IFN signalling late in infection (discussed in Chapter 6).

For many respiratory viruses, IFN $\alpha\beta$ and IFN λ have been shown to be redundant for host protection. Replication of IAV, RSV and HMPV were similar in infected wild type, IFN $\alpha\beta$ R $^{-/-}$ and IFN λ R $^{-/-}$ mice. In contrast, dramatic loss of virus control occurred only in the absence of both IFN $\alpha\beta$ R and IFN λ R receptors (Crotta et al.; Mordstein et al.; Mordstein et al.). From the findings presented in this chapter one could suggest IFN λ to be a viable treatment option for many respiratory viruses, not just IAV. However, IFN λ is not the ideal treatment for all viral infections. When compared directly, pretreatment with IFN α was more effective against Encephalomyocarditis virus (ECMV) and LCMV, while IFN λ pretreatment was shown to be more protective against herpes simplex virus type 2 (HSV-2) infection (Ank et al., 2006a). Furthermore, pretreatment of mice with IFN λ did not alter hepatotropic virus induced disease progression (Mordstein et al.). Differences in effectiveness of IFN treatment to ameliorate virally induced disease is intimately linked to virus tissue tropism. What makes IFN λ 2 treatment effective in IAV infection is that it does not stimulate immune cells, however by the same token this is what makes it ineffective at controlling viruses which can replicate in immune cells. To rephrase, what is nonessential and potentially dangerous IFN $\alpha\beta$ stimulation of the immune system in one virus setting, may be protective through induction of ISG and possibly inflammation in other viral infections. Importantly, we have investigated the use of IFN α and IFN λ as

treatments for severe IAV in a setting where there is a complete and healthy immune system, perhaps immunocompromised individuals would benefit from immune cell stimulation, and in this setting IFN $\alpha\beta$ could be an appropriate treatment.

Although IAV replication is commonly restricted to AECs, some studies have suggested that specific IAV strains can productively replicate in immune or other cell types. Seasonal IAV strains have been demonstrated to be able to infect AMs and DCs in vitro, however this is either nonproductive or abortive (Perrone et al., 2008; Rodgers and Mims, 1982; van Riel et al., 2011; Yu et al., 2011). There is some evidence for productive infection of human monocyte derived macrophages or DCs with highly pathogenic H5N1 avian flu or 1918 Spanish flu IAV strains (Perrone et al., 2008; Sakabe et al., 2011; Thitithanyanont et al., 2007; van Riel et al., 2011; Yu et al., 2011), however whether this occurs in more relevant cell types, specifically AMs and lung or blood derived DCs remains controversial (Smed-Sorensen et al., 2012; van Riel et al., 2011; Yu et al., 2011). pDCs in particular have been demonstrated to be exquisitely immune to productive IAV infection, which is attributed to their potent IFN producing abilities (Thitithanyanont et al., 2007). If immune cell types do allow for productive IAV infection this may limit the effectiveness of IFN λ treatment. Our results indicate that it is likely that AECs are the primary reservoir for IAV replication, hence IFN λ 2 treatment was able to control virus load as well as IFN α 4 (Figures 27 and 32). The majority of IAV NP staining was observed in CD45⁻ cells (Figure 35), although a small proportion of IAV-NP positive immune cells (CD45⁺) was observed. As we are only supplementing the immune response, rather than replacing it entirely with IFN treatment, it is possible that endogenous type I IFN and other antiviral

mechanisms are sufficient to assist in the clearance of IAV from immune cells or indeed, protect them from IAV infection. Furthermore, in certain settings DCs and macrophages have been shown to respond to IFN λ stimulation (de Groen et al., 2015; Liu et al., 2011). Further study is required using different strains of IAV, particularly highly pathogenic H5N1 IAV strains, which have most convincingly been shown to be able to infect immune cells, to ascertain if IFN λ is still protective in these settings.

Results from this section strongly indicate that all immune cells are insensitive to IFN λ treatment, yet there are studies that suggest some immune cell types directly respond to IFN λ . In contrast to the data presented here, Ank et al showed that pDCs directly isolated from mouse spleens express ISGs in response to IFN λ stimulation (Ank et al., 2008). It is possible that Flt3L driven culture used in this study to generate pDCs does not support the expression of IFN λ R on pDCs. Human macrophages, neutrophils, T cells and B cells have all been shown to respond to IFN λ invitro, although in the case of B cells and macrophages this response is not identical to stimulation with IFN $\alpha\beta$ and is often only seen when IFN λ acts in concert with another cytokine or immune stimulus such as TLR agonists (Blazek et al., 2015; Dai et al., 2009; de Groen et al., 2015; Egli et al., 2014a; Jordan et al., 2007; Liu et al., 2011). Given the results of these studies we cannot definitively say that IFN λ does not stimulate immune cells, however the data presented in this chapter does clearly demonstrate that IFN λ 2 treatment alone does not induce secretion of proinflammatory cytokines in vivo or in vitro, nor does it enhance cytokine secretion during IAV infection in the pulmonary environment. It is possible that specific immune cells types only express very low levels of IFN λ R and in this way limit their response to IFN λ . As with IFN $\alpha\beta$ R, the

extent of IFN λ R engagement and ligand affinity and half-life directly modulate outcome of ligand binding (Egli et al., 2014b; Kalie et al., 2008), immune cells may only express low levels of IFN λ R and therefore only be able to respond to IFN λ to a certain extent, whether it be induction of antiviral ISGs or secretion of cytokines which would not be elicited otherwise from IFN $\alpha\beta$ signalling. However we found no evidence of this in the results we attained under from our in vitro studies of immune cells.

Consideration must be given to the long-term effects on the host of IFN treatment. As discussed in Chapter 3, type I IFNs are potent immunomodulators that affect many aspects of both the innate and adaptive immune response. For example, in acute viral infections, IFN $\alpha\beta$ can inhibit DC development (Ito et al.), and IFN $\alpha\beta$ can also drive T cell death or sequestration of lymphocytes in lymphoid organs (Bahl et al., 2010; Shiow et al., 2006). Our own results from Chapter 3 and a study conducted by Price et al. demonstrated that Wt 129 mice had lower induction of IAV specific antibodies compared to IFN $\alpha\beta$ R $^{-/-}$ (129) mice post IAV infection (Price et al.). Lower numbers of B cells were recruited to the lung of IFN α 4 treated mice, as compared to Veh Ctrl group. In contrast, IFN λ 2 treatment did not impede B cell recruitment, however human studies found that IFN λ treatment of anti-CD40 or IL-4 stimulated B cells in vitro modestly downregulated IgG4 production and children with a natural mutation in the IFN λ locus had significantly higher antibody titres post measles vaccine (Hummelshoj et al., 2006). It would be interesting to see if treatment with either IFN α 4 or IFN λ 2 translates into lower induction of IAV specific antibodies in this model.

IFNs have also been reported to affect DC and macrophage function. Myeloid DCs matured in the presence of IFN λ 1 elicited lower IL-13 levels from

naive T cells (Dai et al., 2009) and IFN λ 3 treatment of lung DCs enhanced Th1 cytokine secretion over Th2 cytokines in a mouse model for asthma (Koltsida et al.). IFN λ 1 treatment can also enhance human macrophage secretion of IL-12p40 sensitivity to IFN γ , which, interestingly, IFN α negatively impacts (Ling et al., 1985; Liu et al., 2011; Yoshida et al., 1988). Neither IFN α 4 or IFN λ 2 affected IL-12p40, p70 or IFN γ levels in the BAL during IAV infection, however we did not investigate the cellular source of these cytokines, which may be different in different treatment settings. Additionally, the canonical Th2 cytokines IL-13 and IL-4 were found in comparable levels across all treatment groups, however it must be noted that these cytokines are induced in very low levels in IAV infection and therefore effects of IFN treatment on these cytokines may not be detectable, or indeed relevant.

IFN α 4 treated lungs tended to have lower numbers of neutrophils recruited during IAV infection, which likely stems from IFN $\alpha\beta$ skewing of monocyte cytokine secretion to monocyte recruiting factors such as MCP-1 rather than neutrophil recruiting chemokines such as CXCL1 and CXCL2 (Seo et al.). While neutrophils are superfluous for a robust immune response to IAV (Chapter 3), IAV-IFN $\alpha\beta$ mediated blockade of neutrophils leads to a permissive environment for secondary bacterial infection (Schliehe et al., 2015; Shahangian et al., 2009). A recent study conducted by Blazek et al. also found that both murine and human neutrophils expressed IFN λ R and responded to IFN λ stimulation. Importantly, IFN λ treatment blocked neutrophil production of IL-1 β and recruitment via limiting leukotriene B4 receptor 1 (LTB4R1) expression in experimentally induced inflammation models (Blazek et al.). Analysis of cellular recruitment to the lung during IAV infection in our study revealed that IFN α 4, but not IFN λ 2 treatment lowered pulmonary

infiltration of neutrophils, compared to Veh Ctrl. Neutrophil recruitment to a site of inflammation may be stimulus specific and in the case of IAV infection may not rely exclusively on LTB₄R1, hence the inconsistency in results between Blazek et al.'s and our own study. Significantly, a recent study from our lab demonstrates that TRAIL mediated epithelial cell death also allows for bacterial colonisation post IAV infection (Ellis et al.). Treatment with IFN α 4 therefore may also increase the risk of bacterial superinfection, which is particularly significant as IAV-bacterial co-infection is often fatal to the host, indeed evidence of bacterial pneumonia was found in the majority of post mortem reports and samples from the 1918 H1N1 pandemic (Morens et al., 2008). As IFN α 4 treatment but not IFN λ 2 blunted neutrophil recruitment during IAV infection and perhaps more importantly, IFN λ 2 treatment lowered frequency of apoptotic AECs, in the context of bacterial superinfections IFN λ remains a more appropriate treatment for IAV. However, both IFN α and IFN λ treatments have the potential to facilitate bacterial superinfection through blocking IL-1 β . We did not observe an effect on pulmonary concentrations of IL-1 β by either IFN treatment, however IL-1 β was only observed at low concentrations in our model (data not shown). Infection with different strains or IAV or alterations in initial IAV inoculate may reveal an effect of IFN treatment on IL-1 β function in the lung which may alter disease outcome or indeed permissiveness to secondary bacterial infection, further study is required.

Timing and magnitude of IFN $\alpha\beta$ response can directly impact the outcome of IAV induced disease. Previous studies have demonstrated that pretreatment with recombinant IFN $\alpha\beta$ prior to infection with a range of IAV strains was protective in inbred mice (Beilharz et al., 2007; Cilloniz et al., 2012; Tumpey et al., 2007), guinea pigs (Van Hoeven et al.) and ferrets (Kugel et al.). From these studies

and the results presented in Figure 27 we can conclude pretreatment of animals with type I IFN stimulates the antiviral response in host cells, minimizing the ability of the virus to actually infect host cells and therefore decreasing virus burden from the start. Studies conducted by Van Hoeven et al. and Kugel et al. extended IFN $\alpha\beta$ treatment into administration during infection and while these studies did record reduction in viral titres they did not report on increased sensitivity to seasonal IAV, however these as studies primarily focused control of virus replication and parameters such as inflammation and tissue destruction were not assessed. It is interesting to note however, that in infection with a highly pathogenic H5N1 strain of IAV, IFN α treatment of ferrets both prior to infection and at days 1 and 2 post infection did not confer protection, yet in spite of lower viral load in nasal washings of IFN α treated ferrets this group was as susceptible, if not more so, than their vehicle-treated counterparts (Kugel et al.). Furthermore in Beilharz et al.'s study, where mice were treated with varying doses of IFN α throughout IAV infection, moderate oral doses of IFN α were somewhat protective, while animals given high doses of IFN α suffered higher morbidity than placebo controls. Taken together, our own data and these studies demonstrate that IFN $\alpha\beta$ can be protective, if given at the correct time and in the right amount. In contrast, IFN λ 2 treatment protects against severe IAV disease when given as either a pretreatment (Figure 27) or as a treatment given during infection (Figure 32).

Careful consideration was given to the design of our treatment regime: Treatment commences at 2dpi, with the onset of weight loss and repeated at day 4 and 5 post infection. This regime is easily translatable into a human setting: treatment commencing upon presentation of clinical signs of illness such as fever or coughing and is continued if symptoms persist. Treating at an earlier time point,

e.g.: 12 or 24hrs post infection may increase the effectiveness of both IFN treatments, however like attempting to treat prior to infection, without knowing exactly when infection will occur this is impossible to translate into the clinic. Decreasing the number of doses given or lowering the amount of IFN α 4 given per dose did not result in protection (data not shown). Indeed, decreasing the number of doses of IFN λ 2 also decreased protection (data not shown). Moreover, we specifically selected the IFN subtypes IFN α 4 and IFN λ 2 for their comparatively weaker binding properties (Dellgren et al., 2009; Lavoie et al., 2011). Studies have demonstrated that low IFN binding affinity leads to induction of antiviral ISGs, while high affinity also results in induction of inflammation and associated ISGs (Jaitin et al., 2006; Kalie et al., 2008; Lavoie et al., 2011). We therefore decided it would be safer to select low rather than high affinity IFN subtypes such as IFN β , which may more easily induce inflammation and cell death. Although we have attempted to define a comparable dosing system between IFN α 4 and IFN λ 2, we cannot account for the differing kinetics in downstream signalling. Some studies report that IFN λ signalling elicits a more sustained ISG response than IFN α (Maher and DeStefano), however this may disappear in the context of a viral infection (Jilg et al., 2014). Potentially, a regime could be designed where type I IFN treatment is protective against severe IAV induced disease, in a dose window below pathogenic concentration but above the threshold for ISG induction. However this seems superfluous, as evolution has provided a targeted system which may not have the potential to elicit dangerous side effects. We conclude that the regime we have designed offers the least pathogenic impact for maximal antiviral benefit.

How well this study will translate into clinical application remains to be seen. A caveat of mouse models is that unlike humans, which spend a lifetime

being infected with a menagerie of pathogens, experimental animals are naive to most infectious agents. The immune system is dynamic and is constantly changing to combat or accommodate new stimulus. An obvious example is adaptive immunity: for IAV, weak cross protection between strains has been recorded for both the IAV specific B cell response and the cytotoxic T cell response (Brandenburg et al., 2013; Ekiert et al., 2009; Lee et al., 2008; Tan et al., 2012) (DiLillo et al., 2014) (Mozdzanowska et al., 1999) (Boon et al., 2004; Carragher et al., 2008; LaMere et al., 2011). As discussed, IFN α and to a lesser extent IFN λ have immunomodulatory abilities that can positively or negatively affect adaptive immune responses. Treatment with exogenous IFNs could block or enhance pre-existing immunity to IAV in humans. Indeed with its superior ability to stimulate the adaptive immune system, IFN α could be a better treatment option, although a stronger adaptive immune response may still not be enough to negate IFN α mediated tissue damage. Natural mutations in genes that are connected to IFN signalling, for example IRF7 and IFITM3 (Ciancanelli et al., 2015; Everitt et al., 2012; Zhang et al., 2013), could hamper IFN λ treatment effectiveness, while natural mutations that enhance STAT1 activity (Yamazaki et al., 2014) could over activate IFN signalling, regardless of IFN type. In addition, studies in HCV patients have revealed that humans can be segregated into specific genotypes based upon SNPs in their IFNL locus. HCV infected individuals with genotypes that code for IFN λ 4 protein have a comparatively poor response to IFN α based treatment and lower rates of spontaneous clearance of HCV (Prokunina-Olsson et al., 2013), yet higher baseline ISGs compared to HCV infected individuals who do not code for IFN λ 4. The mechanism behind IFN λ 4 antagonism of other IFNs is currently unknown, however it has been hypothesised that IFN λ 4 may induce a negative

feedback mechanism through prolonged signalling and in particular, induction of USP18 (Egli et al.). It is estimated that 40% of Caucasians express IFN λ 4, therefore it is of interest to see if this SNP alters effectiveness of IFN λ treatment in IAV infection. Careful clinical studies must be carried out to ascertain whether the results presented here are translatable into a human system. Data collected in my lab assessing human PBMC response to IFN λ alone or with IAV indicate that this cytokine does not elicit secretion of proinflammatory cytokines such as IL-6, IP-1, MCP-1 etc., as IFN α does (Data not presented here as it is to be included in Teresa McCabe's thesis). Furthermore, clinical trials of IFN λ in hepatitis C positive patients indicate pegalyted IFN λ is as effective as pegalyted IFN α , however does not induce as severe side effects (Muir et al., 2014).

In conclusion, the data presented in this chapter strongly indicate IFN λ as a preferential IAV treatment option and suggests that the use of IFN α must be considered with caution. IFN α acts on all cell types and is therefore capable of driving host immunopathology. In contrast, the match of IFN λ R expression and IAV tissue tropism allows IFN λ s to target cell types at risk of infection, effectively inducing antiviral genes in these cells and therefore assisting in the control of IAV spread, without the risk of stimulating the immune system to enhance immunopathology.

Chapter 5. Contribution of IFNAR1 to the type I IFN response

5.1 Background

There is strong evidence for a role for host genetic determinants in the outcome of IAV induced disease in humans. As mentioned in previous chapters, SNPs in genes involved in IFN signalling such as STAT1 and IRF7 can markedly affect IAV disease outcome and IAV severity can be clustered into family groups within a population (Albright et al.; Horby et al., 2010; Olsen et al., 2005). Severity of 129 mouse strain IAV induced disease was dependent upon a host intrinsic excessive type I IFN response (Davidson et al., 2014)(Chapter 3). Intriguingly, inter-individual differences in response to type I IFN stimulation in human PBMCs has been recorded (Schlaak et al., 2002).

Although type I IFN subtypes exhibit differential activities and broad range of potencies (Lavoie et al.), they all initiate signalling by binding to the same receptor (IFN $\alpha\beta$ R) that is composed of two subunits known as IFNAR1 and IFNAR2. Each receptor subunit contains an extracellular ligand binding domain and an intracellular kinase domain, and dimerization of IFNAR1 and 2 elicits a phosphorylation cascade, primarily mediated by STAT molecules, to induce ISG expression (Darnell et al., 1994). The quality of antiviral, antiproliferative and immunomodulatory effects of type I IFNs are a function of ligand affinity and density of receptor engagement, however this is a complex relationship which researchers are only beginning to appreciate. Certain type I IFN subtypes can be ISGs themselves, the IFN $\alpha\beta$ R thereby providing a positive feedback loop (Marie et al., 1998). Sensitivity of cells to type I IFNs can be negatively regulated at the receptor level by ubiquitination, endocytosis and degradation of IFNAR1 (Kumar et al., 2007; Kumar et al., 2003; Zheng et al., 2011). Downregulation of IFN $\alpha\beta$ R leads to a refractory period during which cells are nonresponsive to type I IFN

(Huangfu et al., 2012; Qian et al., 2011; Zheng et al., 2011). Importantly, if IFN $\alpha\beta$ R is not downregulated, IFN $\alpha\beta$ signalling can persist and lead to tissue damage {Bhattacharya, 2014 #449}.

Individuals with Down's syndrome (DS) have a total or partial triplication of chromosome 21. Of particular interest, IFNAR1 and IFNAR2 are among many genes encoded by chromosome 21. In 1980, Epstein et al. reported increased sensitivity of Trisomy 21 monocytes to IFN α stimulation in vitro (Epstein et al., 1980), and more recently, stimulation of whole blood from DS patients revealed a higher type I IFN response to influenza virus, as compared to sibling controls (Broers et al., 2012). This in vitro data is complemented by epidemiological studies that show that Down Syndrome sufferers exhibit a higher risk of severe influenza induced disease (Perez-Padilla et al., 2010).

As suggested by the results present in Chapter 3, there may be subsets of humans with genetically determined propensities for an exaggerated IFN $\alpha\beta$ response. Inter individual differences in the IFN $\alpha\beta$ response could be genetically coded for in a myriad of ways, variations in availability, activity or affinity of IFN $\alpha\beta$ subtypes, signalling molecules or transcription factors which initially induce IFN $\alpha\beta$ expression (e.g.: IRF7) or those that are downstream of IFN $\alpha\beta$ R engagement (e.g.: STAT1, TYK2, etc.), reactivity or frequency of IFN $\alpha\beta$ -producing cells such as pDCs or responsiveness of the IFN $\alpha\beta$ R itself could all result in a predisposition for increased IFN $\alpha\beta$ responsiveness, as seen in 129 and DBA mouse strains. We hypothesise that patients with DS represent a genetically defined population whose immune cells have been shown to make increased amounts of IFN $\alpha\beta$ in response to IAV (Broers et al., 2012; Epstein et al., 1980). In this population heightened IFN levels and responsiveness may be causally linked to higher IAV

severity. Identification of genetic markers for susceptibility to IAV would allow for better assessment of 'at risk' individuals within the population. Given the intriguing link between DS, IFN $\alpha\beta$ and IAV severity and our previous data from 129 mice we decided to focus our investigation into one particular gene: IFNAR1.

5.2 Hypothesis and Aims

In this chapter I aimed to build upon the results of my previous work on 129 and B6 mouse strains and their divergent IFN $\alpha\beta$ response to IAV infection. We decided to investigate whether strain specific differences in the gene coding for one of the IFN $\alpha\beta$ R subunits, namely IFNAR1, or the number of IFNAR1 alleles could affect type I IFN levels during IAV infection and consequentially, disease severity.

I hypothesised:

- Decreasing IFNAR1 allele copy number in 129 mice through heterozygous breeding would decrease IFN $\alpha\beta$ levels induced by IAV infection and therefore host morbidity and mortality.
- IFNAR1 activity and or cell surface expression is different between 129 and B6 strains.
- Triplication of the IFNAR1 allele will increase IFN $\alpha\beta$ pulmonary concentrations induced by IAV infection and this will lead to downstream pathology.

5.3 Results

5.3.1 Decrease in IFNAR1 allele number decreases type I IFN response in 129 mice.

In order to assess whether or not IFNAR1 copy number affected IFN $\alpha\beta$ response and IAV disease outcome in 129 mice, we crossed wild type 129 and IFN $\alpha\beta$ R $^{-/-}$ (129) mice. The resulting IFNAR1 $^{+/-}$ (129) mice were phenotypically normal and exhibited no gross abnormalities in vital organs. IFNAR1 heterozygosity slightly reduced the level of IFNAR1 staining on splenocytes, as compared to wild type controls (Figure 36A). Interestingly, reducing the number of functional IFNAR1 alleles decreased the number of pDCs in the lung at baseline, yet did not affect presence of any other cell types (Figure 36B). IFNAR1 $^{+/-}$ (129) mice showed 50% mortality when infected with X31, as compared to the 100% survival of IFN $\alpha\beta$ R $^{-/-}$ (129) mice and 0% of 129 mice (Figure 37A). Intermediate morbidity and mortality correlated to intermediate frequencies of pDCs being recruited to IFNAR1 $^{+/-}$ (129) lungs, during X31 infection. Surprisingly, IFNAR1 $^{+/-}$ (129) mice had an equivalent frequency of IMc recruitment as their wild type counterparts. Neutrophil recruitment was comparable between 129 and IFNAR1 $^{+/-}$ (129) lungs whereas neutrophil recruitment was higher in IFN $\alpha\beta$ R $^{-/-}$ (129) mice, as previously reported (Chapter 3). Unexpectedly, NK cell recruitment in IFNAR1 $^{+/-}$ (129) lungs mimicked frequencies observed in IFN $\alpha\beta$ R $^{-/-}$ (129), not wild type lungs. B cell frequencies were equivalent throughout infection between all three genotypes, with the exception of a trend for more B cells in IFN $\alpha\beta$ R $^{-/-}$ (129) lungs 7dpi. This is in contrast to data reported in Chapter 3, however may be a reflection of the comparatively fewer number of time points taken. Presence of AMs and T cells were comparable between the three genotypes (Figure 37B). IFNAR1 $^{+/-}$ (129)

Figure 36

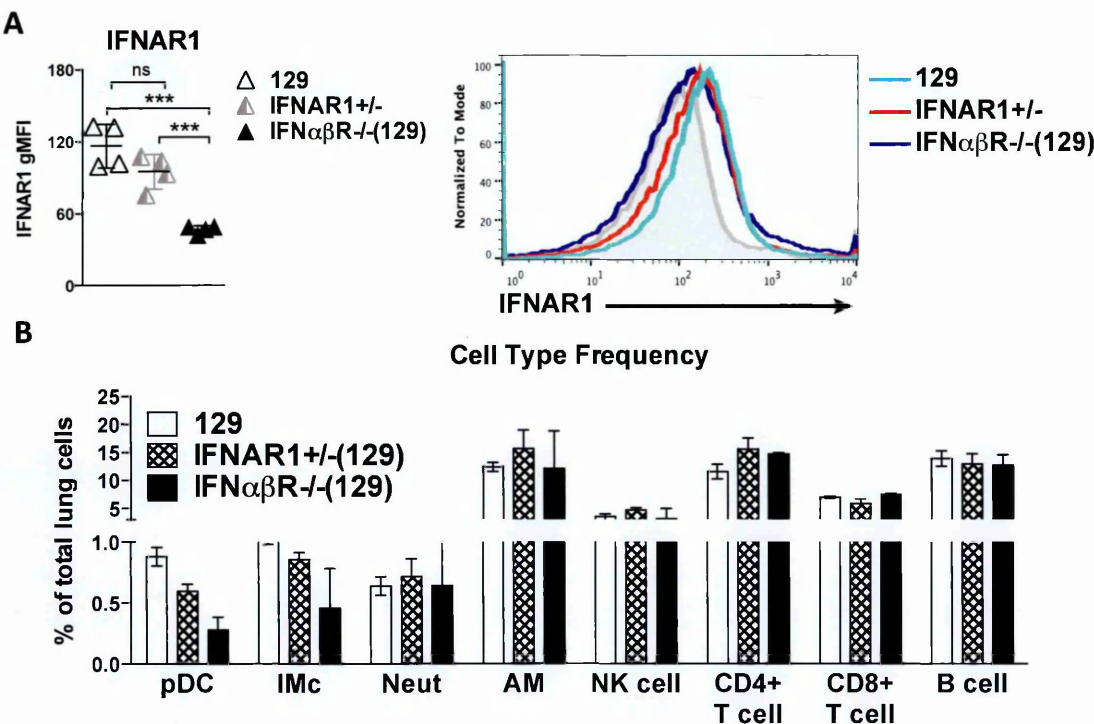


Figure 36: Immune cell subtype frequencies and expression of IFNAR1 in IFNAR1+/- (129) mice. Naïve 129, IFN α β R+/- and IFN α β R-/- mice were sacrificed and **(A)** Whole splenocytes were analysed for IFNAR1 expression by flow cytometry. Column graph shows Mean Fluorescence Intensity (MFI) of IFNAR1 on 129 (open triangles), IFNAR1+/- (grey half filled triangles) and IFN α β R-/(129) (black triangles) splenocytes. Histogram shows 129 mice shown in light blue, IFN α β R+/- in red, IFN α β R-/- in dark blue and unstained control in grey (filled histogram). **(B)** Assessment of frequency of stated immune cell types in the lung were characterised by flow cytometry. Graphs are representative of 2 independent experiments where n=2-3. Significance was assessed by or Student's t test where *** denotes P < 0.001.

Figure 37

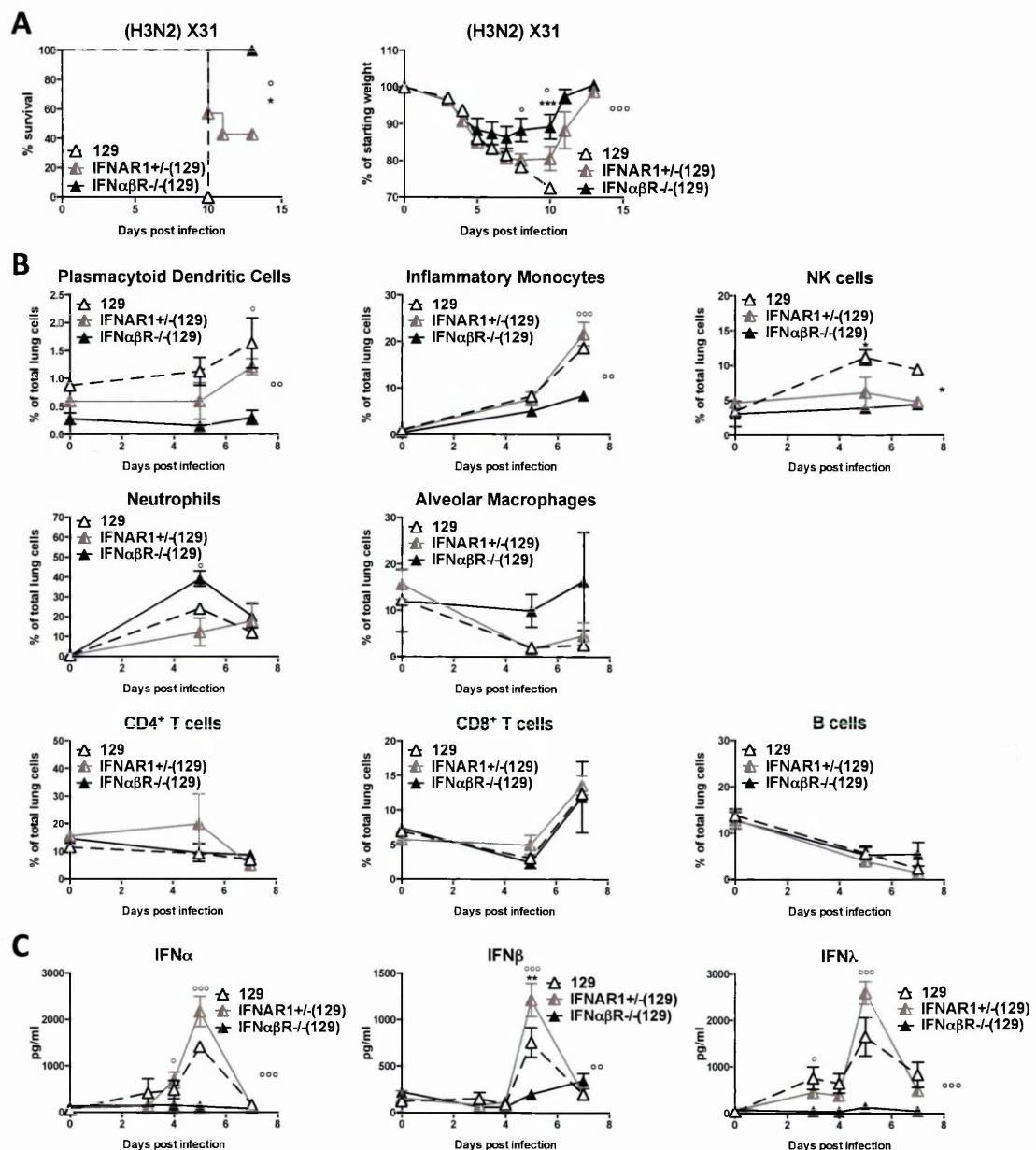


Figure 37: IFNAR1 heterozygosity decreases disease severity of the 129 strain in X31 infection. 129 (open triangles), IFN α BR \pm (grey half filled triangles), IFN α BR \pm (black triangles) were infected with X31 (800TCID₅₀). (A) Weight loss and survival was assessed throughout infection and (B) immune cell recruitment to the lung was assessed by flow cytometry. (C) Levels of IFN α β and λ in BAL of infected mice were assessed by ELISA. Significance assessed by Log-rank (Mantel-Cox) test (survival) and 2-way ANOVA (weight loss and time courses). *, P < 0.05; **, P < 0.01; ***, P < 0.001 where * denoted 129:IFNAR1 \pm -(129) and ° indicates IFNAR1 \pm -(129):IFN α BR \pm -(129) Symbols on the right of graphs indicate statistical significance of the whole curve) and those above indicate significance of individual time points (Bonferroni post test). Graphs show mean \pm SEM and are representative of 2 independent experiments where n=2-6.

mice had higher concentrations of IFN α , β and λ in their BAL fluid than IFN $\alpha\beta$ R $^{-/-}$ (129) mice throughout infection. Interestingly, the concentrations of IFN α , β and λ observed in IFNAR1 $^{+/-}$ (129) lungs were equivalent to, if not higher than what was observed in 129 lungs at days 4 and 5 post infection (Figure 37C). These results indicate that decreasing IFNAR1 allele copy number reduces IAV induced pathology in the 129 strain. However, this may be an effect of a de novo mutation within one either the 129 or IFN $\alpha\beta$ R $^{-/-}$ (129) parental strains and completely unrelated to IFNAR1. An intercross between IFNAR1 $^{+/-}$ (129) mice should be performed to determine whether or not degrees of susceptibility to IAV induced disease segregate based upon the IFNAR1 locus. Although, genetic overlap between parental strains has been previously confirmed by microsatellite analysis (Charles River, data not shown).

5.3.2 Strain differences in IFNAR

Having observed that IFNAR1 allele number can alter disease outcome, we then went on to assess if there was a strain specific difference in IFNAR1 between 129 and B6 mice. A myriad of factors could be at work upstream or downstream of IFNAR1 that could contribute to 129 augmented responsiveness to IFN $\alpha\beta$. Differences in IFNAR1 or IFNAR2 affinity to IFN $\alpha\beta$ subtypes or indeed strain specific differences in the IFN $\alpha\beta$ subtypes secreted in response to IAV infection or the affinity of these subtypes themselves could all contribute to 129 susceptibility and in turn be blunted by a decrease in IFNAR1 allele frequency. Additionally, signalling downstream of IFN $\alpha\beta$ R such as receptor subunit affinity to Tyk2 and Jak1, availability of signalling molecules or readiness of any of these components to be phosphorylated could also alter IFN $\alpha\beta$ signalling. Finally, level of IFN $\alpha\beta$ R

expression on the cell surface and down regulation upon ligand engagement could also lead to differences between B6 and 129 in IFN $\alpha\beta$ signalling magnitude and or duration. We hypothesised that IFNAR1 has different cell surface expression levels and downregulation/re-expression kinetics that may alter IFN $\alpha\beta$ signalling. To study this, we first compared the coding sequence between 129 and B6 IFNAR1. Sequencing of 129 and B6 IFNAR1 coding region did not reveal any difference between strains (data not shown), confirming indications in the Sanger database. However, IFNAR1 expression could be modulated by external factors such as availability of tonic IFN $\alpha\beta$ signal or strain specific differences in the IFNAR1 promoter locus. We therefore decided to compare IFNAR1 expression, downregulation and reappearance on the cell surface between 129 and B6 strains.

In vitro stimulation of whole splenocytes with IFN $\alpha 4$ over 24hrs was performed and IFNAR1 expression on immune cells was assessed. As reported in the literature, IFNAR1 expression was down regulated upon stimulation in both 129 and B6 splenocytes. At resting, there was a slight trend for higher expression of IFNAR1 on 129 cells (Figure 38A), however down regulation post ligand engagement and later reappearance of IFNAR1 molecules was comparable between 129 and B6 whole splenocytes cultures (Figure 38B). Interestingly, when splenocytes were loosely divided into 'lymphoid' (cells expressing CD3+ or CD19+) and 'myeloid' (cells expressing CD11c+ and/or CD11b+) cell types, it appeared 129 myeloid cells had a significantly higher expression of IFNAR1 which was down regulated in a shorter time period and expression returned to baseline at a more rapid rate, compared to B6 myeloid splenocytes. No difference in kinetics was observed for the lymphoid population (Figure 38C).

Figure 38

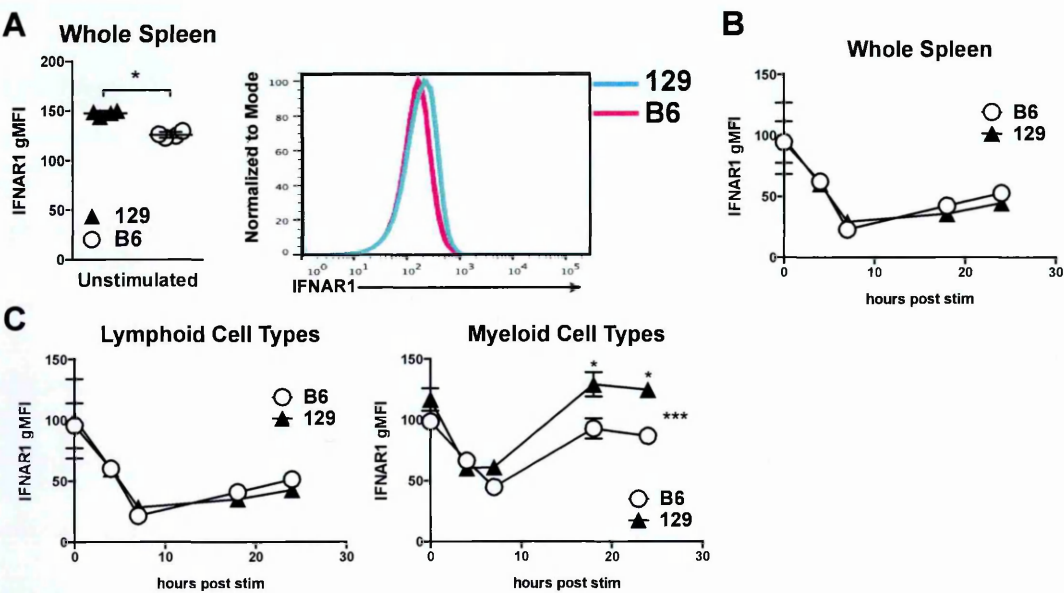


Figure 38: IFNAR1 is down regulated post stimulation with IFN α 4. 129 and B6 whole splenocytes were stimulated with IFN α 4 (1000U/ml) and analysed for IFNAR1 expression by flow cytometry at specified time points. (A) Baseline expression of IFNAR1 on 129 (triangles) and B6 (circles) on whole splenocytes, as assessed by MFI. Histogram shows 129 mice shown in blue and B6 in pink IFNAR1 baseline expression. (B) IFNAR1 MFI on 129 and B6 whole spleonocyte cultures post IFN α 4 treatment, (C) splenocytes were also separated into myeloid (CD11b+ and or CD11c+) and lymphoid (CD19+ or CD3+) populations and IFNAR1 MFI was graphed. Significance assessed by student's T test (unstimulated) or 2-way ANOVA (time courses). *, P < 0.01; ***, P < 0.001 where symbols on the right of graphs indicate statistical significance of the whole curve and those above indicate significance of individual time points (Bonferrioni post test). Graphs are representative of 1 experiments where n=2-4.

This data indicates there could be strain specific differences in IFNAR1 between 129 and B6 mice that may alter the type I IFN response. To investigate further, we bred (129xB6)F1 mice and infected them with X31. Similar to what was observed in Figure 37 with IFNAR^{+/-}(129) mice, (129xB6)F1 mice were intermediately susceptible to IAV induced disease, in the context of 100% survival of B6 mice and the 0% survival of the 129 strain (Figure 39A). (129xB6)F1 lungs exhibited intermediate levels of IFN α and IFN λ (compared to the high responding 129 and low responding B6 strains) early in infection. However at 5dpi concentrations of IFN α and IFN λ in (129xB6)F1 lungs were comparable to what was observed in 129 mice. In contrast, concentration of IFN β in the (129xB6)F1 lung was comparable to B6 levels (Figure 39B).

We wanted to probe whether or not the intermediate phenotype observed in (129xB6)F1 mice was related to number of IFNAR1 alleles from the 129 background, similar to what was observed in IFNAR1^{+/-}(129) mice. F1 breeding generates offspring which are 50% 129 and 50% B6 which means, similar to inbred laboratory mouse strains, F1 mice are genetic copies of one another. We therefore decided to take advantage of this to specifically look at differences between IFNAR1 from the 129 and the B6 background. As shown in Figure 40 we bred Wt 129 mice with IFN $\alpha\beta$ R^{-/-}(B6) mice to generate F1 mice where the only functional allele for IFNAR1 was of 129 origin (IFNAR1 129 F1 mice). Similarly, we bred wild type B6 mice with IFN $\alpha\beta$ R^{-/-}(129) mice to yield mice where the only functional copy of IFNAR1 was from the B6 strain (IFNAR B6 F1 mice). Comparison of immune cells in naive lungs revealed comparable frequencies of all cell types tested between IFNAR1 129 F1, IFNAR1 B6 F1 and (129xB6)F1 strains. Differences in pDC frequency in 129 and B6 lungs at resting, as was as previously

Figure 39

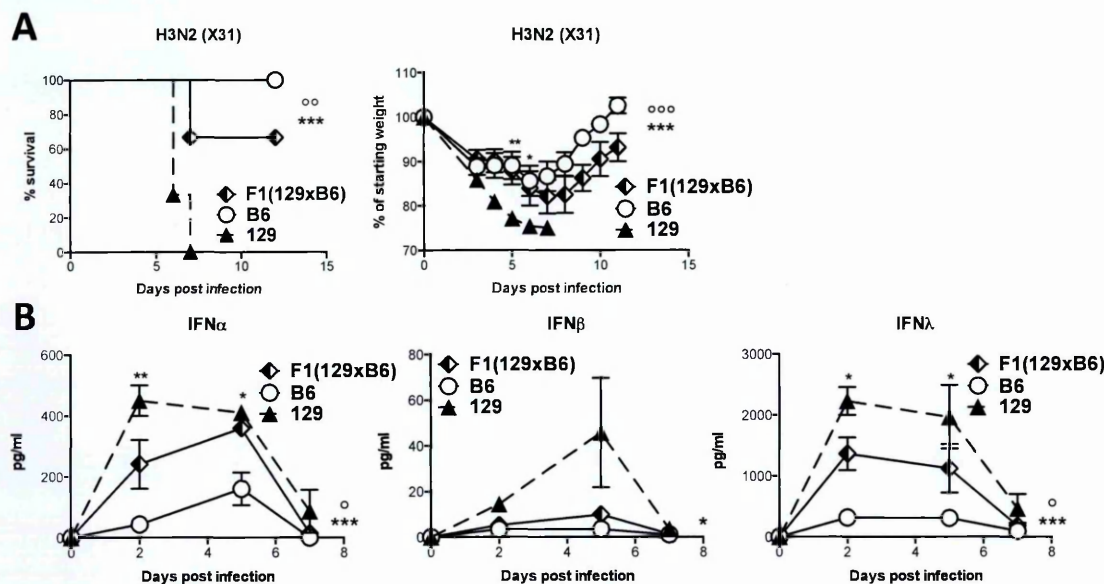


Figure 39: Intermediate concentrations of IFN α in (129xB6)F1 lungs early in X31 correlate to intermediate morbidity and mortality to X31 induced disease. 129 (triangles), B6 (open circles) and (129xB6)F1 (half filled diamonds) mice were infected with X31 (800TCID₅₀). (A) Weight loss and survival were recorded through out infection. (B) At specified time points BAL fluid concentrations of IFN α , β and λ were assessed by ELISA. Graphs show mean \pm s.e.m and are representative of 2 independent experiments where n=2-6. *** p<0.0001, ** p<0.001, * p<0.01 by 2-way ANOVA with Bonferroni post tests (weight loss and IFN concentration) or Log-rank (Mantel-Cox) Test (survival) where * denotes 129:B6, ° 129:(129xB6)F1, B6:(129xB6)F1 was not significant. Symbols on the right of graphs indicate statistical significance of the whole curve and those above indicate significance of individual time points (Bonferroni post test).

Figure 40

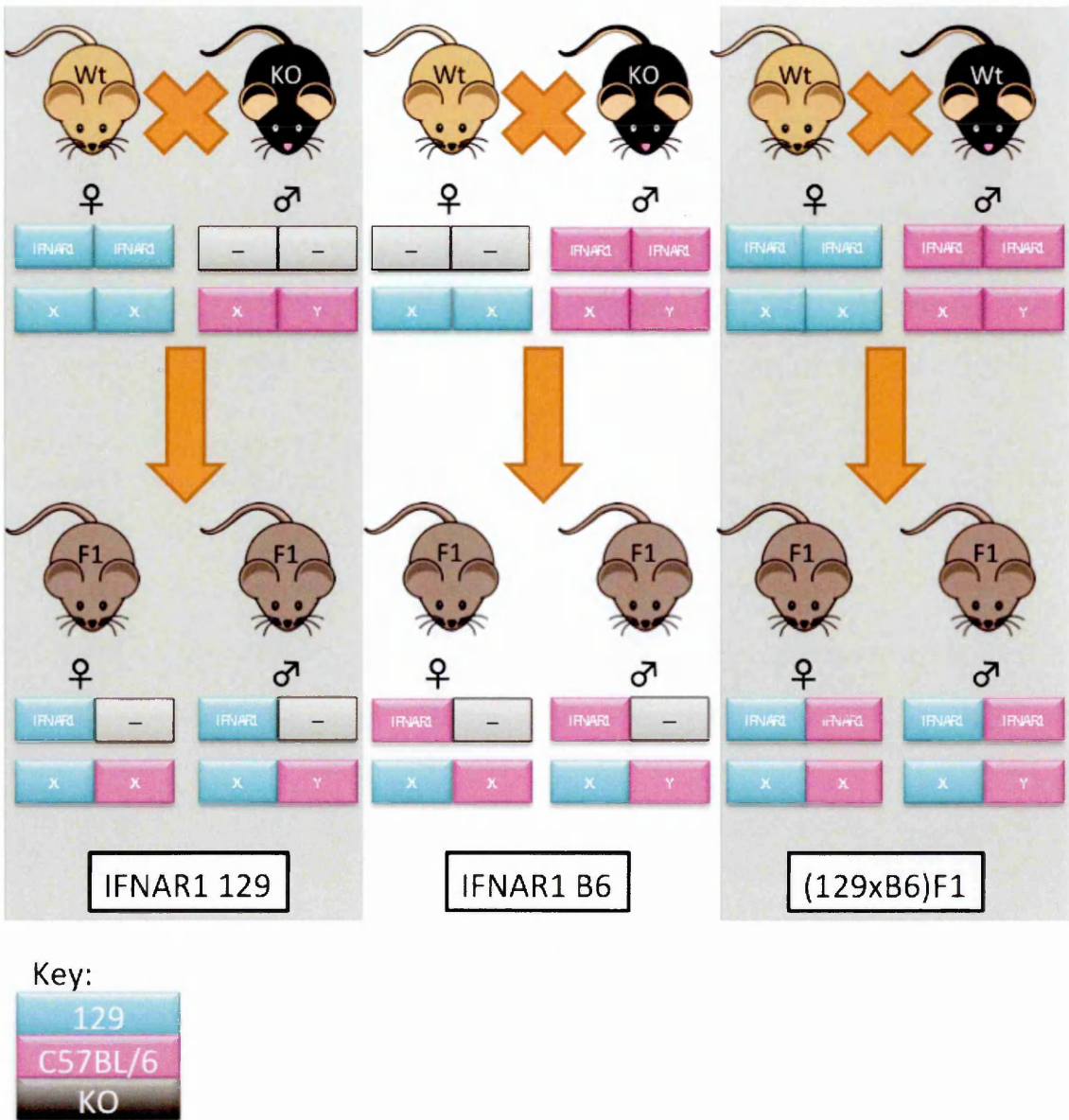


Figure 40: Breeding scheme for carriage of IFNAR1 from either 129 or B6 parental strain in (129xB6)F1 mice. Wild type 129 female mice were bred with male IFN $\alpha\beta$ R $^{-/-}$ (B6) mice to generate an F1 generation where only the IFNAR1 alleles from the 129 parental strain is functional, called IFNAR1 129. Similarly, IFN $\alpha\beta$ R $^{-/-}$ -(129) female mice were bred with wild type B6 males, resulting in progeny that have only a B6 IFNAR1 functional allele: IFNAR1 B6 mice. For control mice, wild type 129 females were bred with wild type B6 males: (129xB6)F1 mice.

Figure 41

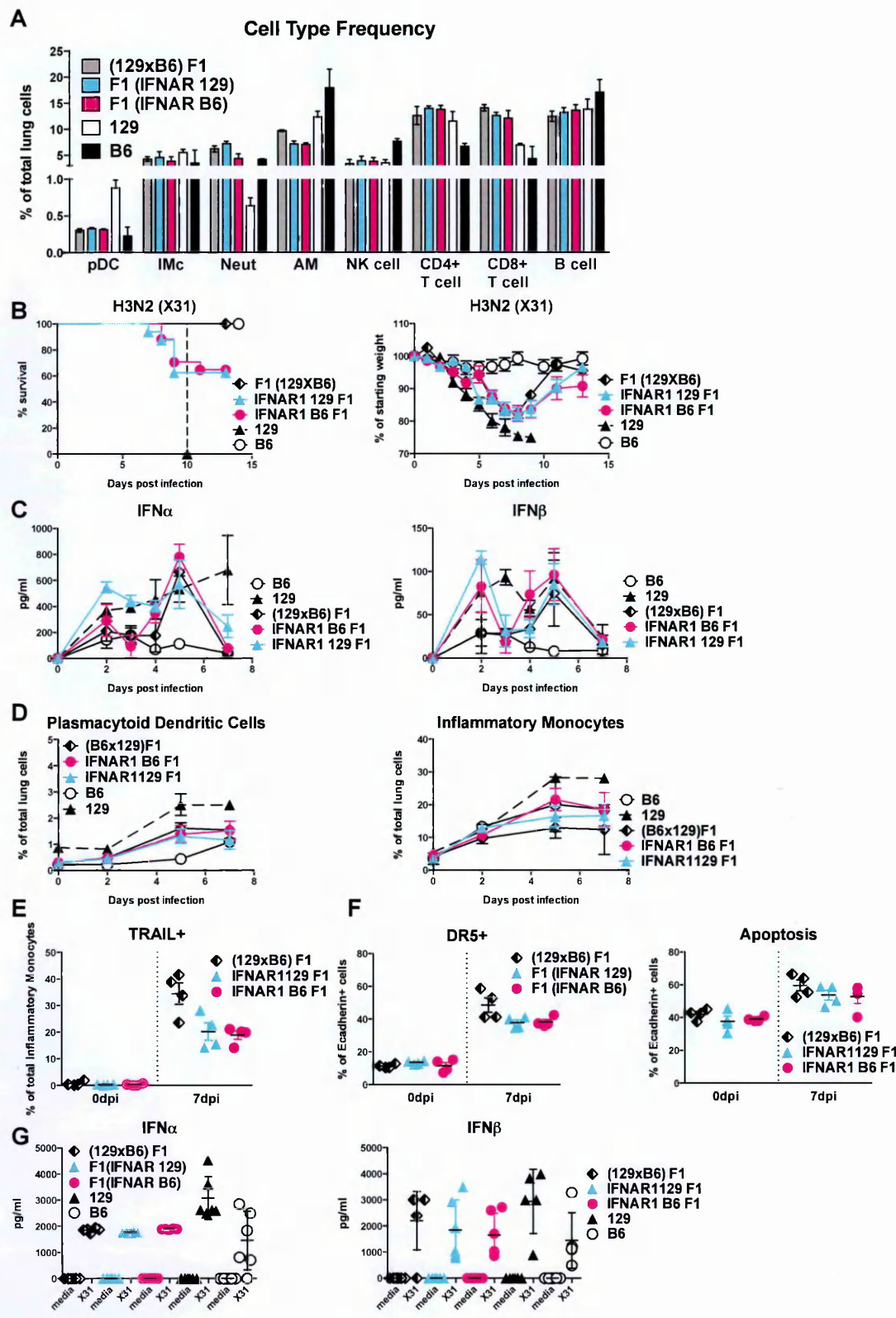


Figure 41: Strain origin of IFNAR1 does not alter X31 induced disease course in (129xB6)F1 mice. (A) Frequency of stated immune cell types in naïve lungs of 129 (white bars), B6 (black bars), IFNAR1 129 F1 (blue bars), IFNAR1 B6 F1 (pink bars) and

reported (Chapter 3), was observed, while copy number or strain origin of IFNAR1 allele did not appear to affect pDC frequency in mice on the F1 background (Figure 41A).

Infection with X31 (800TCID₅₀) of IFNAR1 129 F1 and IFNAR1 B6 F1 strains along with control (129xB6)F1, 129 and B6 strains with X31 revealed that IFNAR1 129 F1 and IFNAR1 B6 F1 mice were equally susceptible to IAV induced disease. IFNAR1 129 F1 and IFNAR1 B6 F1 mice exhibited increased morbidity and mortality compared to B6 and interestingly, (129xB6)F1 mice. As expected, 129 mice were most susceptible to IAV induced disease (Figure 41B). Closer investigation revealed that strain origin of IFNAR1 did not alter IFN α or β concentrations within the BAL at any time point assessed, with IFNAR 129 F1, IFNAR B6 F1 and (129xB6)F1 lungs all having comparable concentrations of IFN α and β . IFN $\alpha\beta$ levels in 129 lungs were not significantly higher than those observed in F1 strains in this experiment, however as expected, concentrations of IFN α and β in B6 lungs were comparatively low (Figure 41C). IFNAR1 strain origin did not alter pDC or IMc recruitment to the lung during infection, nor did it augment TRAIL expression on IMcs, epithelial cell expression of DR5 or frequency of

Figure 41 (cont): (129xB6)F1 (grey bars) mice were characterised by flow cytometry. (B-F) 129 (black triangles), B6 (open circles), IFNAR1 129 F1 (blue triangles), IFNAR1 B6 F1 (pink circles) and (129xB6)F1 (half filled diamonds) mice were infected with X31 (800TCID₅₀) and (B) survival and weight loss was assessed throughout infection. (C) Levels of IFN α and β in BAL of infected mice were assessed by ELISA and (D) flow cytometry was used to assess pDC and IMc recruitment to the lung, (E) IMc TRAIL expression and (F) epithelial cell expression of DR5 and death. (G) Flt3 derived pDCs from stated mouse strains were stimulated with X31 (MOI: 1) for 24hrs and supernatants were analysed by ELISA for IFN α and β . Significance was assessed between IFNAR1 129 F1 and IFNAR1 B6 F1 as well as compared to control (129xB6)F1 strain, however no statistically significant differences were found.

epithelial cell death in the lung (Figure 41D-F). Finally, BM derived pDCs generated from IFNAR1 129 F1, IFNAR1 B6 F1 and (129xB6)F1 mice responded with identical levels of IFN α , β and λ post stimulation with live X31 (Figure 41G).

This data clearly demonstrates that restricting the origin of IFNAR1 to one strain (either 129 or B6) in (129xB6)F1 mice does not alter the disease course during X31 infection, however in the context of an (129xB6)F1 background having only one functional IFNAR1 allele can increase disease sensitivity (although this was not statistically significant).

5.3.3 Triplication of IFNAR1 and IAV infection

Genes encoding for human IFN $\alpha\beta$ R subunits are located on chromosome 21, PMBCs from DS patients make more IFN $\alpha\beta$ when stimulated with IAV than sibling controls and DS monocytes are more sensitive to IFN $\alpha\beta$ signalling (Broers et al., 2012; Epstein et al., 1980). Intriguingly, individuals with DS exhibit a higher risk of severe IAV induced disease (Perez-Padilla et al.). There is clear correlation, yet no causal link established between influenza susceptibility and the IFN-related hyperresponsiveness in DS individuals. Although triplication of chromosome 21 leads to triplication of a hundreds of genes present on this chromosome genes coding for the IFN $\alpha\beta$ R are excellent candidates to explain this increased IFN $\alpha\beta$ responsiveness. Genes on human chromosome 21 have murine homologues spread across mouse chromosomes 10, 16 and 17, with IFNAR1 and IFNAR2 being coded for on murine chromosome 16 (Cox et al., 1984; Sheppard et al., 2012). We hypothesise that trisomy of the IFNAR locus in DS suffers leads to increased IFN $\alpha\beta$ responsiveness and this responsiveness leads to IFN $\alpha\beta$ mediated immunopathology in IAV infection. To assess this we employed

Figure 42

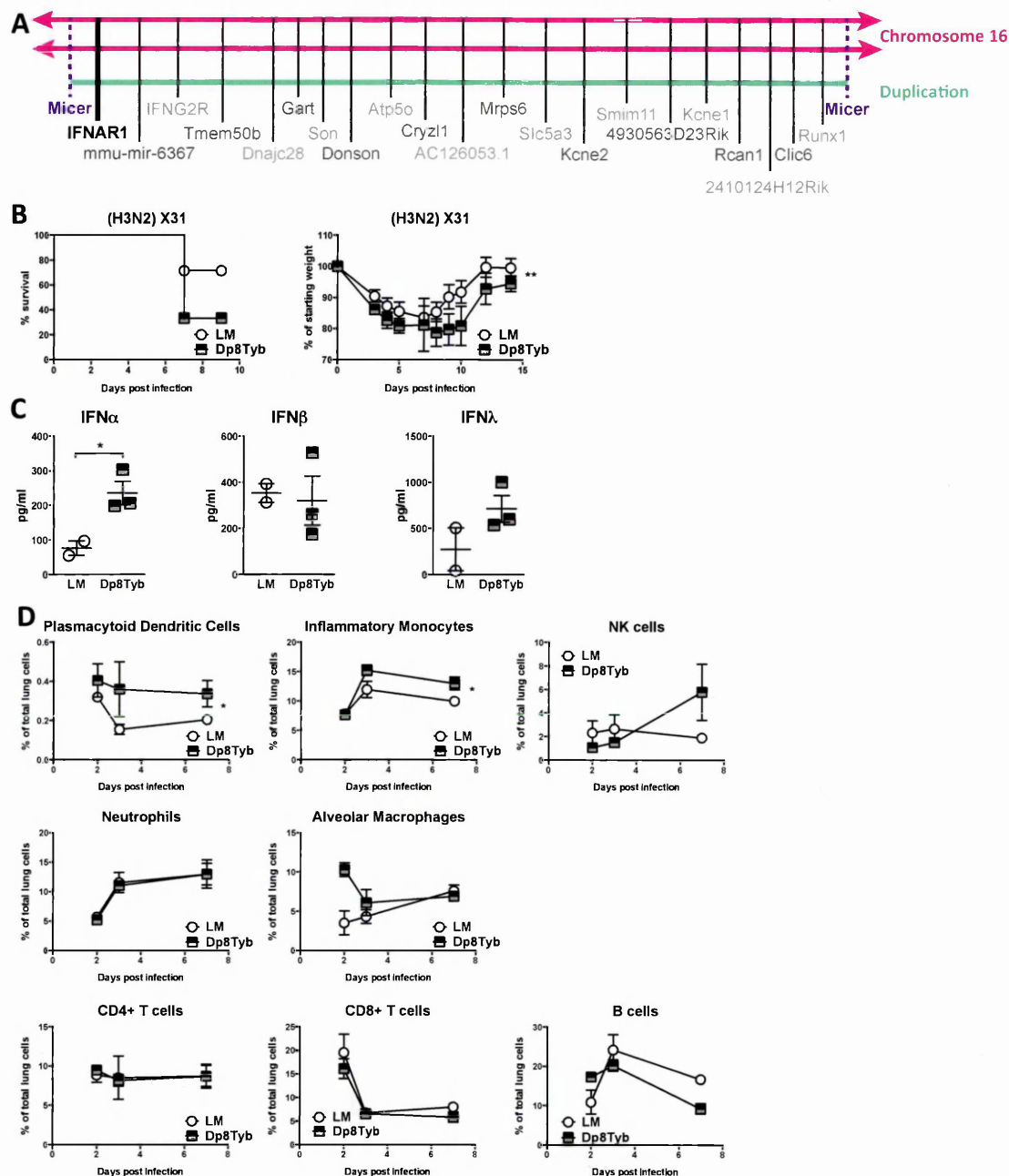


Figure 42: Triplication of a region of Chromosome 16 containing *IFNAR1* in B6 mice enhances pulmonary levels of *IFNα* and IMc recruitment during X31 infection. (A) Genes in Dp8Tyb duplicated region, green line defines duplication, diagram is representative of relative gene positions, however is not to scale. (B-D) Dp8Tyb mice (black and grey squares) and littermate controls (LM) (open circles) were infected (X31: 800TCID₅₀) and (B) survival and weight loss was assessed throughout infection. (C) Levels of *IFNα*, *β* and *λ* in BAL at 3dpi were assessed by ELISA and (D) recruitment of specified cell types to the lung analysed by flow cytometry. Significance assessed by Log-rank (Mantel-Cox) test (survival), 2-way ANOVA (weight loss and time courses) or Student's t test (*IFN*). *, *P* < 0.05; **, *P* < 0.01. Symbols on the right of graphs indicate statistical significance of the whole curve. Graphs show mean ± SEM and are representative of 1 experiment where n=3-8.

Dp(16Ifnar1-Runx1)8TybEmcf/B6 mice, hereby called Dp8Tyb mice which carry duplication of a section of mouse chromosome 16 between the genes IFNAR1 and Runx1(16:91436064 to 16:93062456), a region of approximately 21. These mice were generated in the laboratory of Dr. Victor Tybulewicz (Francis Crick Institute, Mill Hill) and are currently unpublished. Duplication of this region in Dp8Tyb mice leads to these 21 genes being present in one extra copy, ergo Dp8Tyb mice have 3 copies of these 21 genes (Figure 42A). Dp8Tyb mice are bred on the B6 background, are phenotypically normal and exhibit no gross abnormalities in vital organs. In particular, no abnormalities were observed in thymic or cardiac tissue (data not shown).

Upon X31 infection Dp8Tyb mice trended to experience increased morbidity and mortality (Figure 42B). Excitingly, the increase in mortality correlated to higher concentrations of IFN α in the BAL at 3dpi in Dp8Tyb mice, as compared to IFN α concentration observed in littermate controls (Figure 42C). Additionally, higher frequencies of pDCs and IMcs were recruited to the lungs of Dp8Tyb mice throughout infection, as compared to Dp8Tyb littermate controls. NK cell frequency was also elevated in Dp8Tyb mice at 7dpi. All other cell types assessed were found in comparable frequencies in the lungs of Dp8Tyb and littermate control mice at the time points assessed, with the exception of B cells which were recruited in lower numbers to the lung in mutant mice late in infection (Figure 42C). Collectively this data suggests that triplication of IFNAR1 can lead to increased sensitivity of IFN $\alpha\beta$ signalling which can negatively impact IAV disease outcome.

5.4 Discussion

The contribution of host genetics to the outcome of human disease is increasingly being appreciated. Susceptibility to severe seasonal or pandemic IAV in humans is likely to be polygenic and co-determined by pathogen characteristics, immunological memory, co-morbidities and environmental factors. It would be naive to assume a single gene is the cause of the massive difference observed between 129 and B6 inbred mouse strains. However, results in this chapter do indicate that while the coding sequence of the IFNAR1 gene itself does not differ between 129 and B6 genomes, IFNAR1 may be regulated differently between these strains and importantly, increasing or decreasing the number of IFNAR1 alleles can alter IAV disease outcome.

Not unlike what was observed in Chapter 3 with genomic ablation of IFNAR1 in 129 and B6 strains IFNAR1, heterozygosity conferred increased or decreased resistance to IAV disease severity, depending on host background. (129xB6)F1 mice with only one functional copy of IFNAR1 (IFNAR1 129 F1 and IFNAR1 B6 F1 mice) exhibited higher mortality than their (129xB6)F1 controls. In contrast, IFNAR1^{+/-} mice on the pure 129 background were less susceptible to IAV induced disease than their wild type counterparts. We hypothesise that decreasing IFNAR1 allele number decreases responsiveness to IFN $\alpha\beta$ and on a pure 129 background and this is protective, however on a (129xB6)F1 mixed background lowering IFN $\alpha\beta$ responsiveness may be suboptimal for induction of the antiviral state. Expression of TRAIL on IMcs and DR5 on AECs in F1 mice with only one IFNAR1 allele is lower, implying that IFNAR1 129 F1 and IFNAR1 B6 F1 mice may be less responsive to IFN $\alpha\beta$ signalling. Therefore, IFNAR1 129 F1 and IFNAR1 B6 F1 mice may have elevated IAV loads in their lungs compared to the (129xB6)F1

strain and this could increase IAV induced disease burden, however as we did not assess viral loads in the experiments presented in Figure 41 this hypothesis requires further analysis. Indeed, to extend this study IFNAR1+/- mice on the B6 background should also be generated, it would be interesting to determine if IFNAR1+/- (B6) mice also exhibited an intermediate phenotype as IFNAR1+/- (129) mice do. Furthermore for better clarity of virus control this hypothesis could be tested in the presence of an intact Mx1 gene by using mice which code for a functional Mx1 protein for the generation of F1 mice. This hypothesis aside, it is unlikely that the effects we observe in strains derived from F1 breedings are entirely mediated by a single gene, it is likely many genomic factors contribute to differences in cell type frequency, responsiveness to IFN $\alpha\beta$ and indeed, outcome of IAV infection, in any setting including, a mixed genetic background such as (129xB6)F1 mice.

Interestingly, increased resistance of IFNAR1+/- (129) mice associated with decreased frequencies of pDCs in their lung at baseline and a trend for blunted pDC recruitment to the lung compared to Wt mice during X31 infection. As discussed in Chapter 3, pDC development from CLPs is severely impeded without IFN $\alpha\beta$ R signalling (Chen et al., 2013) and IFN $\alpha\beta$ can be a survival factor for mature pDCs (Kadowaki et al., 2000). A reduction in the number of IFNAR1 alleles could negatively impact IFN $\alpha\beta$ R signalling and therefore pDC development and survival on the 129 background, ultimately leading to better resistance to IAV induced immunopathology. In contrast, pDC frequency in naive IFNAR1+/- F1 (IFNAR1 129 F1, and IFNAR1 B6 F1) mice was equivalent to baseline frequency of pDCs in (129xB6)F1 lungs. By crossing low pDC frequency B6 mice with a high pDC frequency strain: NOD2 mice, Pelletier et al. found regulation of the pDC

compartment size is multigenic: primarily regulated by a locus on mouse chromosome 7 (named: Pdcc1) and modified by loci on chromosomes 9 and 11. In this study the IFNAR locus was not identified to contribute to pDC compartment size and propensity of pDCs to make IFN $\alpha\beta$ in response to stimulus was not linked to any genetic loci (Pelletier et al., 2012). IFNAR1 allele copy number may affect pDC compartment size in a pure 129 background however, in the context of a genome that is 50% B6, loss of one functional allele does not alter pDC frequency in the lung. Interestingly, the Flt3L gene is encoded within the Pdcc1 region (Gene, 2015). Sequence alignment of available 129 strains to the reference C57BL/6 (Jackson) strain in the Sanger database found several SNPs in the 129 strains, yet none were within Flt3L coding region (data not shown). However this does not exclude differences in expression regulation of the locus between strains. It is possible that Flt3L encoded in the 129 genome may act synergistically with the increased sensitivity to IFN $\alpha\beta$ R signalling observed in the 129 strain to increase pDC frequency in this strain. Genetic deletion of one IFNAR1 allele may lower sensitivity to IFN $\alpha\beta$ and thereby lower pDC frequency in IFNAR1 $^{+/-}$ (129) mice. However, in the context of (129xB6)F1 mice where the B6 Flt3L allele (or indeed the entire Pdcc1 locus) may be dominant, modification of pDC compartment size does not occur.

While IFNAR1 heterozygosity in 129 mice decreases the frequency of pDCs in the lung during IAV infection, it did not negatively impact IFN, β and λ concentration in the BAL, particularly late in infection. Indeed, at 5dpi, IFNAR1 $^{+/-}$ (129) lungs contained higher levels of IFN $\alpha\beta$ and IFN λ compared to their 129 controls. Previous data presented in this thesis strongly demonstrates high concentrations of IFN $\alpha\beta$ can lead to tissue damage and therefore host mortality.

Yet, in spite of higher IFN $\alpha\beta$ pulmonary concentrations IFNAR1+/- (129) mice are more resistant to IAV induced disease than their wild type counterparts. Reduction of IFNAR1 expression on cell surface may decrease receptor availability, thereby lowering IFN $\alpha\beta$ signal transduction. So although IFN $\alpha\beta$ concentrations in the BAL fluid of IFNAR1+/- (129) mice are equivalent to what is observed in Wt 129 mice, the actual amount of downstream signalling in IFNAR1+/- (129) cells is lower due to a reduced number of IFN $\alpha\beta$ R complexes able to transduce the signal. Furthermore, a lower frequency of IFN $\alpha\beta$ R being available for ligand engagement may change the quality of IFN $\alpha\beta$ signalling. Low levels of IFN $\alpha\beta$ R ligand engagement on human carcinoma cells has been shown to result in induction of ISGs associated with antiviral genes, while increased receptor engagement results in expression of immunomodulatory and apoptotic ISGs such as MCP-1 or TRAIL (Kalie et al., 2008; Lavoie et al., 2011). In conclusion, although there are higher concentrations of IFN $\alpha\beta$ in the IFNAR1+/- pulmonary environment, decreasing IFNAR1 expression may act as a bottleneck, where IFN $\alpha\beta$ signal is decreased as a whole, or receptor engagement is only sufficient to induce expression of antiviral ISGs. Further investigation into proinflammatory cytokine levels and TRAIL expression in IFNAR1+/- (129) lungs during IAV infection is required.

In the context of a comparatively more resistant background, specifically the (129xB6)F1 background, reduction of IFNAR1 alleles to one increased host susceptibility to IAV induced disease. This increase in susceptibility occurred regardless of IFNAR1 strain origin (129 or B6). Coupled with confirmation that the coding sequence of IFNAR1 from the 129 and B6 genomes are identical, we can conclude that there is unlikely to be a biological difference in influenza resistance associated with the 129 versus B6 IFNAR1 locus. Even so, the increase in

susceptibility exhibited by both IFNAR1 129 F1, and IFNAR1 B6 F1 is of interest. Similar to what we found in the IFNAR1+/- (129) mice, decreasing the number of IFNAR1 alleles did not affect X31 induced levels of IFN $\alpha\beta$ in the BAL fluid, as compared to the (129xB6)F1 controls. In contrast to IFNAR1+/- (129) mice, pDC numbers and recruitment were not affected by the decrease in IFNAR1 alleles. Furthermore, bone marrow derived IFNAR 129 F1 and IFNAR B6 F1 pDCs secreted IFN α , β and λ in amounts comparable to (129xB6)F1 pDCs. Thus, in line with what was observed in IAV infection in vivo, having only one functional IFNAR1 allele does not impede IFN production by pDCs on the (129xB6)F1 background. Finally, TRAIL expression on IMcs and DR5 expression on AECs were lower in the IFNAR1 129 F1 and IFNAR1 B6 F1 strains, this is suggestive of decreased IFN $\alpha\beta$ R availability lowering the magnitude or quality of IFN $\alpha\beta$ signalling (as discussed above). What is therefore causing increased host pathology in these strains compared to the (129xB6)F1 strain is unknown. It is possible that less IFNAR1 availability is resulting in decreased induction of antiviral genes, however this is unlikely given studies conducted by the Schreiber group (Kalie et al., 2008; Lavoie et al., 2011), further investigation is required.

Triplication of the IFNAR1 gene may increase IAV sensitivity in an IFN $\alpha\beta$ dependent manner. Dp8Tyb mice, trisomic for a portion of chromosome 16 which contains the gene coding for IFNAR1, are less resistant to IAV induced disease than their littermate controls and exhibit elevated IFN α levels and increased pDC and IMcs numbers in the lung during IAV infection, thus IAV infection of Dp8Tyb mice parallels disease hallmarks seen in 129 mice (Chapter 3). Early experiments conducted by Epstein et al. demonstrated that trisomy 21 cells bind more interferon ligand in fact in accordance with gene dosage, approximately 50% more

(Epstein et al., 1982). As a consequence of increased binding capacity human trisomy 21 monocytes and fibroblasts have increased sensitivity to IFN α stimulation in vitro (Epstein and Epstein, 1976; Epstein et al., 1980). More recently, Broers et al demonstrated that whole blood samples from DS patients make more type I IFNs when stimulated with IAV as compared to their sibling controls, importantly viral titres in these cultures were equivalent (Broers et al., 2012). These studies and the higher concentration of IFN α recorded in Dp8Tyb lungs at 3dpi strongly indicate that DS patients may have an exaggerated type I IFN response to IAV.

Intriguingly, epidemiological data collected during the recent swine flu pandemic indicated likelihood of hospitalization, intubation, and death were 16-fold, 8-fold, and 335-fold greater respectively, for patients with DS than non DS patients (Perez-Padilla et al., 2010). Additionally, DS children have a high frequency of IAV, RSV and other respiratory tract infections, independent of co-morbidity diagnoses of congenital heart disease and asthma (Bloemers et al., 2007; Selikowitz, 1992; Turner et al., 1990). Moreover, DS patients have a higher incidence of acute lung injury secondary to pneumonia, compared to normal control children (Bruijn et al., 2007; Hilton et al., 1999). Thus, severe disease caused by IAV and other respiratory pathogens associates with comparatively strong IFN $\alpha\beta$ reactivity in DS patients and similarly, this is what we observe in Dp8Tyb mice.

It has also been reported that DS individuals are acutely sensitive to sepsis, having a 30% higher risk of fatality, when compared to other patients hospitalized for sepsis, after controlling for confounding factors (Garrison et al., 2005; Hill et al., 2003). The early phase of sepsis is characterized by excessive inflammation and in

some cases cytokine storm (Shukla et al., 2014). IFN $\alpha\beta$ signalling has been intimately linked to LPS induced sepsis (Mahieu et al., 2006), IFNAR1 $^{-/-}$ mice being protected from LPS induced septic shock (de Weerd et al., 2013). Recently, de Weerd et al. demonstrated a novel IFNAR1-IFN β (and IFNAR2 independent) signalling pathway that induces a select set of ISGs, including MCP-1 and Mip1- α , which mediated mortality in a model for LPS induced sepsis (de Weerd et al., 2013). Induction of proinflammatory cytokines by this IFNAR1-IFN β signalling is hinted at by the increased recruitment of IMcs in Dp8Tyb mice. It is possible that the triplication of IFNAR1 allows for increased opportunity for IFNAR2 independent IFNAR1-IFN β signalling and thereby increasing induction of proinflammatory cytokines and therefore IAV induced pathology in Dp8Tyb mice.

Although we attribute Dp8Tyb mouse and DS patient susceptibility to IAV to an elevated IFN $\alpha\beta$ response many studies associate DS patient sensitivity to respiratory infections with depressed cellular and/or humoral immunity. Defective T-cell maturation is an early integral feature of DS (Burgio et al., 1978), normal expansion of naive helper and cytotoxic T lymphocytes is lacking in the first years of life, although T cell populations gradually reach comparable levels with healthy controls (de Hingh et al., 2005; Kusters et al., 2010). Reduced T cell numbers in DS children is attributed to abnormal thymus development and reduced thymic output (Bloemers et al., 2011). Similarly, primary expansion of B cells seen in healthy children early in life was severely abrogated in DS sufferers yet unlike T cells, B cell numbers remain severely decreased, all stages of peripheral B-cell development being altered in DS, with more severe defects seen during the later stages of B-cell development (Carsetti et al., 2015; de Hingh et al., 2005). In addition, analysis of serum pre and post vaccination with IAV or Streptococcal

pneumonia vaccines of DS patients demonstrated that while antibody induction did occur, it was comparatively lower than healthy controls (Costa-Carvalho et al., 2006; Kusters et al., 2012). Whether B cell defects are linked to decreased T cell help, is intrinsic to B cells themselves or is the result of other external factors remains unknown (Verstegen et al., 2010). In contrast to human data, the T cell response in IAV infected Dp8Tyb mice was shown to be equivalent to littermate controls. CD4⁺ and CD8⁺ T cells were recruited in comparable frequencies, however assessment of an IAV specific cytotoxic T cell response, or indeed control of IAV replication was not performed so we cannot comment on whether these aspects contributed to IAV susceptibility.

B cell recruitment was blunted in Dp8Tyb lungs, whether or not this is linked to trisomy of IFNAR1 or another gene in the triplicated region or if this reduced recruitment results in diminished induction of IAV specific antibodies requires further investigation, however it is remarkably reminiscent of the decreased B cell recruitment observed in 129 mice. Additionally, we did not assess baseline differences in B cell subtype frequencies so we cannot comment on whether or not these are affected by triplication in this model. However, early recruitment of B cells at days 2 and 3 post infection in Dp8Tyb mice are comparable to litter mate controls. This hints at events induced by infection, such as IFN $\alpha\beta$ secretion triggering in some way a reduction of the B cell population in the lung, this may be through blockade of B cell recruitment to the lung, apoptosis of B cells present in the lung or perhaps modulation of B cells in other organs such as the spleen or bone marrow. In contrast to experimental mice, humans are constantly exposed to immune stimulus and this may provide a tonic signal for IFN $\alpha\beta$ expression, individuals with DS with their increased propensity to respond

to IFN $\alpha\beta$ (Broers et al., 2012; Epstein et al., 1980) may therefore be more sensitive to this tonic signal and thus suppression in B cells numbers may be a result of constant elevated IFN $\alpha\beta$ levels. This may not be observed in mice kept in specific pathogen free conditions, as exposure to varied immune stimulus does not occur in this setting and therefore our hypothesised negative effects of IFN $\alpha\beta$ on B cells may only manifest in the context of infection.

Given that the Dp8Tyb triplication is only of a small section of mouse chromosome 16 we do not expect to recapitulate all aspects of DS pathology, indeed defects in adaptive immunity seen in DS patients are largely not observed in these mice. Defects in trisomic T and B cells may contribute to severity of IAV induced disease in DS patients, however as observed in 129 mice, IFN $\alpha\beta$ mediated pulmonary tissue death and therefore pathology occurred in spite of a intact adaptive response, therefore making study of IFNAR1 and the type I IFN response in DS patients during IAV infection important. Undoubtedly, there is the distinct possibility that other genes triplicated in Dp8Tyb mice contribute to disease severity, within this region there are several genes for proteins involved in ion channel flux: CLIC6, KCNE1 and KCNE2, several genes coding for structural and chaperone proteins: DNAJC28, ATP50, ITS1 and SMIM11, as well as SON which regulates cell cycle and pre-messenger RNA splicing, RUNX1 which is involved in the development of normal hematopoiesis and in particular, appears to support mature CD4⁺ and CD8⁺ T cells in the spleen and finally, IFNGR2 which is the high affinity subunit of the type II IFN receptor (Barro-Soria et al., 2014; Chen et al., 1995; Dergai et al., 2010; Friedli et al., 2003; Gardiner et al., 2002; Khan et al., 1994; North et al., 2004; Tinel et al., 2000; Zhang et al., 2008). In addition, genes coding for IFNAR2 (the high affinity subunit of IFN $\alpha\beta$ R) and IL10RB (the low

affinity receptor chain for not only IL-10R but also IFN λ R) are coded for in close proximity to the Dp8Tyb region and may also be affected by the triplication, indeed there may be as yet undefined interactions between genes within the area. Moreover, these genes are found in analogously on human chromosome 21 and therefore, particularly IFNAR2 could contribute the elevated IFN $\alpha\beta$ responsiveness observed in DS suffers. Investigation into the Dp(16Mis18a-Runx1)2TybEmcf/B6 (triplication: 16:90719312 to 16:92826066) and Dp(16Mis18a-Il10rb)7TybEmcf/B6 (triplication: 16:90719312 to 16:91425834) mouse strains which have triplications that encompass both the genes coding for IFNAR1 and IFNAR2 and only for IFNAR2 (respectively) would allow us to understand if this increase in IFN $\alpha\beta$ signalling is specific to IFNAR1 triplication or is achieved by either IFNAR1 or IFNAR2 and whether or not triplication of the whole receptor complex enhances this phenomenon.

Potentially, all genes triplicated in Dp8Tyb mice and those surround this region could contribute to the increase in severity of IAV infection in Dp8Tyb mice. Alterations in genes coding for ion channel flux may lead to cardiac defects, however susceptibility to virus induced pathology in DS patients has been shown to occur independent of congenital heart disease (Bloemers et al., 2007; Selikowitz, 1992; Turner et al., 1990). It is also conceivable that triplication of IFNGR2, as we hypothesise for IFNAR1 could increase IFN γ signalling and thereby over activate NK cells or macrophages leading to tissue damage (Okamoto et al., 2002). In addition, genes involved in hematopoiesis and cell cycle regulation could contribute to an immune response that is inappropriate to combat IAV infection, leading to host pathology.

Symptoms of DS are multifactorial and dependent not only on the level of chromosome triplication but, like all human disease, genetics of the entire genome, environment, immune history and co-morbidities, how these factors affect the outcome of IAV infection requires in-depth study. While Dp8Tyb mice cannot encompass the entirety of DS, these mice do allow us to focus on IAV induced immunopathology potentially mediated by an augmented IFN $\alpha\beta$ response. Data presented in this chapter strongly indicate changes in IFNAR1 copy number can alter IAV induced disease severity. Future study into Dp8Tyb mice as a model of severe IAV induced disease in DS patients should be directed towards blocking IFN $\alpha\beta$ signalling through antibody blockade of IFNAR1, antibody mediated pDC and/or IMc depletion and breeding of Dp8Tyb mice with IFN $\alpha\beta$ R $^{-/-}$ (B6) mice to ascertain whether decreasing IFNAR1 copy number in Dp8Tyb mice does in fact ameliorate IAV induced disease severity.

Chapter 6. Concluding Remarks and Future Directions

Collectively, the data in this thesis demonstrate that the relationship between IFN $\alpha\beta$ response and IAV resistance can be characterised by a bell shaped curve with highest resistance at intermediate IFN $\alpha\beta$ responses and reduced resistance at very high or very low IFN $\alpha\beta$ responses (Figure 43). Although superficially this may be counterintuitive, it is a common feature of many biological processes. The induction of ISGs through stimulation of cells by IFN $\alpha\beta$ is vital for protection in systemic viral infections (Muller et al., 1994). However, as productive replication of IAV is generally restricted to AECs, a cell type in which IFN λ and IFN $\alpha\beta$ are redundant for induction of the antiviral state, IAV infection is a model in which we can dissect the immunomodulatory action of IFN $\alpha\beta$ independently of its antiviral role. Although IFN $\alpha\beta$ has been implicated in immune mediated pathology in systemic viral infection, particularly chronic infections such as LCMV and HIV, the pathogenic component of IFN $\alpha\beta$ signalling is substantially more difficult to disentangle from protective IFN $\alpha\beta$ antiviral action in initial infection. Indeed, the key finding of this thesis: IFN $\alpha\beta$ pathogenicity in IAV infection, could only be resolved in the context of IFN λ redundancy in AECs.

In Chapter 3 we show by genetic, cell ablation and mAb-blocking experiments that in IAV-infected hosts, excessive amounts of IFN $\alpha\beta$ produced by PDCA-1⁺ cells is upstream of induction of cytokine storm, recruitment of TRAIL⁺ IMcs and upregulation of DR5 on AECs, culminating in high host morbidity and mortality. However, it is important to remember that these events are designed to be protective for the host; secretion of proinflammatory cytokines and chemokines recruit immune cells to control initial virus spread, take up and present antigen, kill and clear away infected cells and eventually resolve viral infection. Although exaggerated IFN $\alpha\beta$ signalling in 129 mice lead to tissue damage, the effects of

Figure 43

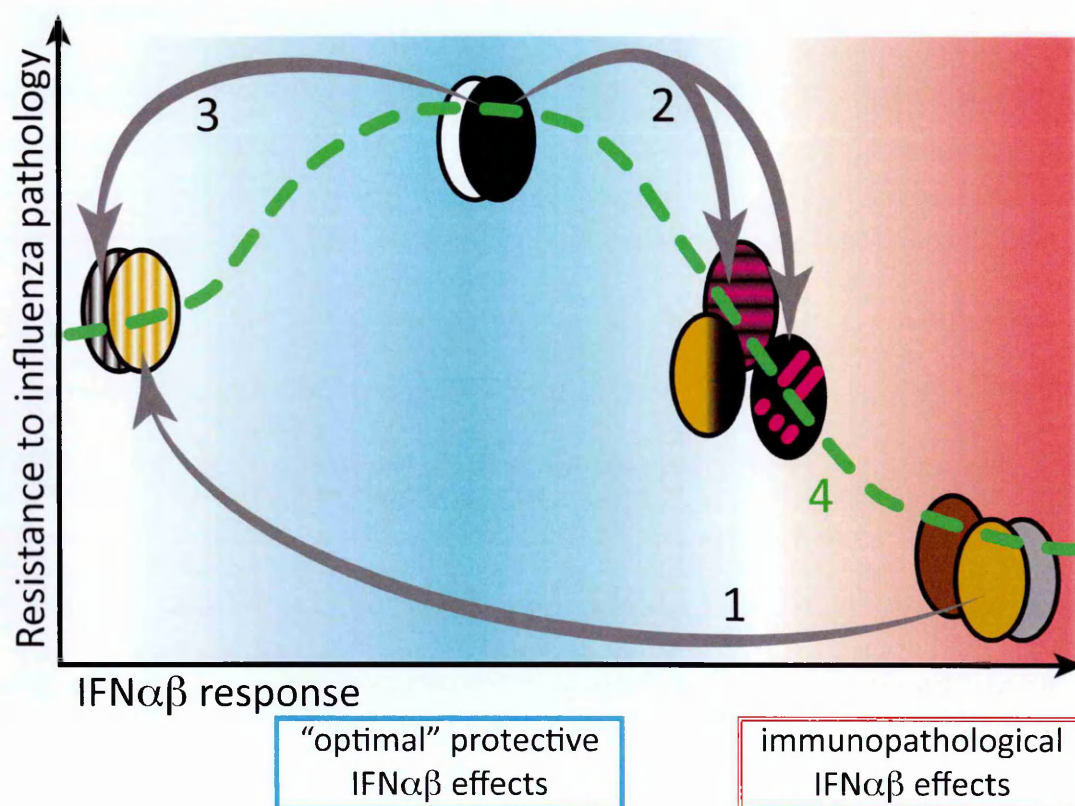


Figure 43: The relationship between IFN $\alpha\beta$ response and resistance to IAV induced pathology is a bell shaped curve. Graphical representation of conclusions drawn and hypotheses made from the data presented in this thesis. Highly IFN $\alpha\beta$ responsive mouse strains: 129 (agouti oval), DBA (grey oval) and CBA/J (brown oval) are acutely susceptible to IAV induced disease, while BALB/C (white oval) and B6 (black oval) strains which produce comparably lower amounts of IFN $\alpha\beta$ in response to IAV infection are resistant. Therefore, moderate IFN $\alpha\beta$ responses to IAV are protective, while excessive IFN $\alpha\beta$ amounts contribute to immunopathology. (1) Complete genetic ablation of IFN $\alpha\beta$ R signalling in 129 mice markedly increases strain increases resistance. (2) Conversely, exogenous addition of IFN α to resistant, low IFN $\alpha\beta$ -expressing mouse strains B6 (or B6.A2G-Mx1 strains) or increasing this strain's responsiveness to IFN $\alpha\beta$ ligands by triplication of IFNAR1 gene (black and pink ovals) drives IAV associated immunopathology in this strain. (3) However, if the moderate IFN $\alpha\beta$ responses in B6 mice are genetically removed, influenza resistance is reduced. (4) Linking these data points generates a dose-response curve where in IAV infection moderate IFN $\alpha\beta$ responses protect and high IFN $\alpha\beta$ responses are detrimental.

IFN $\alpha\beta$ at lower concentrations in B6 mice were protective. Indeed, the mild increase in disease burden in IAV infected IFN $\alpha\beta$ R $^{-/-}$ (B6) mice may be due to lack of IFN $\alpha\beta$ mediated induction of appropriate proinflammatory cytokines leading to preferential recruitment neutrophils over IMcs during IAV (Seo et al., 2011; Sprenger et al., 1996). It is interesting to note that both CCR2 dependent monocytes (including IMcs defined in this study) and TRAIL action in IAV infection have both been described to have both protective and pathogenic roles (Aldridge et al., 2009; Brincks et al., 2011; Brincks et al., 2008a; Dawson et al., 2000; Herold et al., 2008; Hogner et al., 2013; Lin et al., 2008). Given that we delineate a pathway where IMc recruitment and subsequent expression of TRAIL is modulated by IFN $\alpha\beta$, it is unsurprising that we again return to the concept of a bell-shaped curve, where there is an optimal magnitude of CCR2 monocyte recruitment or TRAIL expression and exceeding or falling short of this optimal level of response can lead to host pathology.

Mouse strain comparison in Chapter 3 allowed for the characterisation of immunopathology brought about by host intrinsic high pulmonary concentrations of IFN $\alpha\beta$ induced by IAV infection. The high disease severity experienced by 129 mice during IAV infection associates with significantly elevated concentrations of IFN $\alpha\beta$ in the lung that persist late into infection, compared to the more resistant B6 strain where comparatively lower levels of IFN $\alpha\beta$ are induced by IAV infection and these are only observed during the early phase of infection. While genetic ablation of IFN $\alpha\beta$ signalling did protect 129 mice from severe disease, the question remains is it the peak levels, the overall concentrations or the persistence of IFN $\alpha\beta$ in the lung, or a combination of these, that drives immunopathology? We must ask: Are high levels of IFN $\alpha\beta$ early in infection actually contributing to the downstream

TRAIL/DR5 mediated epithelial cell tissue destruction? Or is this purely the effect of IFN $\alpha\beta$ on the immune environment late in IAV infection? Or is persistent IFN $\alpha\beta$ signalling throughout infection amplifying inflammation and consequently immunopathology? As TRAIL and DR5 expression only appear to be upregulated late in IAV infection it is tempting to assume that persisting concentrations of IFN $\alpha\beta$ are to blame. Indeed IFN $\alpha\beta$ mediated induction of inflammation, regulation of apoptosis and cell cycle arrest require a prolonged period of IFN $\alpha\beta$ signalling (Kalie et al., 2008; Piehler et al., 2000; Roisman et al., 2005). We have demonstrated that repeated exogenous treatment with IFN α of IAV infected B6 mice increases disease burden. Using this model we could directly test the contribution of IFN α concentration and kinetics to IAV induced pathology by comparing large single doses of IFN α at specific time points of infection with prolonged treatment. Concomitantly, could blockade of IFN $\alpha\beta$ signalling late in IAV infection ameliorate severe disease? However, this idea must be approached with caution, as it is likely that within any human population there will be a spectrum of IFN $\alpha\beta$ response profiles (Schlaak et al., 2002) and as observed in IFN $\alpha\beta$ R $^{-/-}$ (B6) mice, blockade of IFN $\alpha\beta$ signalling can also exacerbate disease through increasing host permissiveness to IAV replication.

Based upon our findings in 129 mice and the wider literature we can suggest molecular targets that will lower IFN $\alpha\beta$ levels in an IAV infected host. A key feature of the immune system is redundancy. This is exquisitely demonstrated between RIG-I and TLR7, these signalling pathways offering entirely independent means of IAV recognition and subsequent IFN $\alpha\beta$ induction (Koyama et al., 2007; Pang et al., 2013). RIG-I appears to be the dominant PRR for IAV, it is ubiquitously expressed and most cell types are unable to recognise IAV without it (Kallfass et

al., 2013; Koyama et al., 2007). pDC expression of TLR7 is therefore likely to be an evolutionary fail safe, a second line of defence for when viruses manage to subvert RIG-I recognition, as IAV attempts to do through action of NS1 or when viral infections become systemic. For example, pulmonary infection of IFN α 6 reporter mice with NDV revealed that AMs were the primary producers of IFN α 6 and pDCs did not contribute to this response, however upon systemic NDV infection pDCs were the primary producers of IFN α β (Kumagai et al., 2009). Both pDCs and TLR7 have been demonstrated to be unnecessary for effective resolution of primary IAV infection, as genetic ablation of TLR7 signalling in B6 mice does not alter disease outcome or decrease levels of IFN α β induced in the lung at 24hrs post IAV infection (Jeisy-Scott et al., 2012; Koyama et al., 2007). Two studies have demonstrated that mAb PDCA-1 mediated depletion of pDCs did not alter host mortality during IAV infection (GeurtsvanKessel et al., 2008; Soloff et al., 2012). These studies did however report contradictory results for pulmonary levels of IFN α post pDC depletion; GeurtsvanKessel et al. found no difference while Soloff et al. described lowered IFN α concentrations in the lungs of α PDCA-1 treated mice. The discrepancy between these two studies is likely due to the fact that GeurtsvanKessel et al. focussed on one early time point, while Soloff et al. assessed IFN α level at several time points during infection. Intriguingly Soloff et al. also observed decreased IAV induced weight loss in pDC depleted mice. Importantly, both of these studies were performed in B6 mice, a strain which we have defined in this thesis to be an optimal IFN α β responder. Soloff et al.'s study on pDC depleted B6 mice parallels the results we observe using the same method of pDC depletion in IAV 129 mice. Significantly, depletion of pDCs in both these studies lowered IAV induced pulmonary levels of IFN α and host morbidity, in the context of the low

IFN $\alpha\beta$ producing strain this is only a mild change in disease burden however in the context of the high IFN $\alpha\beta$ responding 129 strain this significantly ameliorates IAV induced disease. Therefore, targeting pDCs and their IAV recognition pathway could allow for the development of a treatment which could be given to an entire population, regardless on an individual's level of IFN $\alpha\beta$ responsiveness. An obvious choice would be to deplete pDCs during IAV infection, however mAbs are an expensive treatment option and as such not desirable for treatment of a large human population.

Alternatively, TLR7 antagonism may blunt IFN α secretion from pDCs and thereby lower pulmonary concentrations of IFN α in individuals with a propensity to make high levels of IFN $\alpha\beta$, consequently protecting these individuals from IFN $\alpha\beta$ mediated tissue damage; yet due to TLR7's redundancy with RIG-I this antagonism should not impede IAV clearance from the lung in any individual, regardless of genotype. However, TLR7 antagonism would only interrupt IFN $\alpha\beta$ secretion directly down stream of pattern recognition but will not affect the IFN $\alpha\beta$ /IFN $\alpha\beta$ R positive feedback loop and therefore this strategy will not control pDC production of IFN $\alpha\beta$ induced by other stimuli, including IFN $\alpha\beta$ R engagement. As AMs, AECs and other DCs are all known to secrete IFN $\alpha\beta$ in IAV infection it is unlikely that TLR7 antagonism would block IFN β secretion from these cell types and therefore no blockade of IFN β -IFN $\alpha\beta$ R feedback would occur on pDCs present in the lung. Another confounding factor which may preclude TLR7 antagonism as a treatment for IAV is that TLR7 has been shown to be indispensable for induction of IAV specific CD4⁺ T cell, B cell and Ab responses (Jeisy-Scott et al., 2012; Koyama et al., 2007). Thus, TLR7 antagonism may lower IFN $\alpha\beta$ mediated tissue damage yet cause pathology through blockade of an appropriate IAV specific adaptive

response. Again, a more appropriate treatment for IAV in humans may be to deplete pDCs.

Although convincing, the data discussed above is based purely in inbred laboratory mouse strains in a limited number of IAV infection settings. Therefore these studies may not account for circumstances where pDCs and TLR7 are vital as a fail-safe mechanism. This could be in individuals whose IFN $\alpha\beta$ responsiveness is significantly below what is optimal or in cases where the cellular tropism of IAV infection is not restricted to AECs. Significantly, as IAV NS1 can block signals required for IFN induction (Diebold et al., 2003; Kallfass et al., 2013), infection of immune cells could significantly decrease cell types able to secrete IFN $\alpha\beta$ and a rapid response from pDCs may be essential for host survival. Furthermore, Kaminski et al. demonstrated that comprising TLR7 signalling or depleting pDCs in a highly pathogenic H7N7 infection of Mx-1 functional mice partially increased disease severity (Kaminski et al., 2012). In conclusion, host-directed therapies that stimulate antiviral activity against IAV in order to resolve the infection more rapidly may be preferential to blockade features of the immune system.

As demonstrated in Chapter 4, IFN λ may be a viable option for broad-spectrum treatment of IAV in immunocompetent humans, although further research is required. As mentioned in the discussion of Chapter 4, expanding the strains of IAV tested is vital to ascertain whether or not IFN λ is a suitable treatment for all IAV strains, even strains which have the potential to infect immune cells. Translation of this model into ferrets would be an important step since these animals are considered a more clinically relevant model for IAV due to their sialic acid moieties on their AECs (Jayaraman et al., 2012). Additionally, as ferrets are a well-established model for monitoring IAV transmission between

hosts, the effectiveness of IFN λ to block transmission in these animals could also be assessed (Herlocher et al., 2001). Excitingly, IFN λ as a therapy in humans has already passed early safety trials and has been assessed in clinical trials as a treatment for HCV and hepatitis B virus (HBV) infection in humans. Reminiscent of our own results, this trial reported lower side effects from IFN λ treatment compared to IFN α treatment (Muir et al., 2014). Of course further study on the human immune cell response to IFN λ and clinical testing to determine whether or not IFN λ is effective at lowering IAV induced disease burden, particularly with respect to not eliciting a cytokine storm in humans, is required. Yet tantalising preliminary results attained in my lab with human PMBCs stimulated with IFN λ do show that IFN λ alone does not elicit ISG expression or proinflammatory cytokine secretion and importantly, IFN λ does not augment cytokine secretion from these immune cells when given with IAV.

The translational capacity of these results notwithstanding, further investigation using the mouse model is warranted. It would be of interest to employ a similar method to Mahlakōiv et al. who stained for Mx1 protein in the guts of IFN α or IFN λ treated mice. Using this technique the authors were able to elegantly show that unlike the lung, the gut has a compartmentalized IFN system, in which epithelial cells primarily respond to IFN λ , while other cell types in the gut rely on IFN $\alpha\beta$ for antiviral defence (Mahlakoiv et al., 2015). Staining of lung histology slides for Mx1 protein in IFN α 4 and IFN λ 2 mice may allow us to identify pulmonary immune cells that respond to IFN λ . Another interesting experiment is to generate bone marrow chimeras made from B6.A2G-Mx1 mice reconstituted with either an IFN $\alpha\beta$ R sufficient or an IFN $\alpha\beta$ R deficient hematopoietic system and to treat these mice with IFN α 4 during IAV infection. If the conclusions we draw

from the data presented in chapter 4 are correct and it is only the action of IFN α 4 on immune cells which drives cytokine storm, innate cell recruitment and immune mediated tissue damage, then chimeras generated with an IFN α β R $^{-/-}$ immune system should be protected, while chimeras generated with a Wt hematopoietic system that can respond to IFN α 4 would suffer severe disease (as observed in whole Wt mice treated with IFN α 4 during IAV infection). By restricting the cell types able to respond to IFN α 4 treatment to nonhematopoietic cells it is possible that we recapitulate the targeted effect of IFN λ treatment in whole mice. However, there are two major foreseeable caveats in this experiment: Firstly, as was observed in chimeras generated in 129 mice, radioresistant myeloid cells may still cause epithelial cell apoptosis, however as previously mentioned, the B6 strain appears more robust than the 129 strain in terms of tissue repair or resistance to damage. Indeed, similar chimeras generated by Hogner et al., specifically Wt B6 mice reconstituted with either an IFN α β R $^{-/-}$ or a TRAIL $^{-/-}$ hematopoietic system, were protected from IFN α β -TRAIL mediated epithelial cell death (discussed in depth in Chapter 3) (Hogner et al., 2013), so the hardiness of the B6 strain may compensate for IFN α 4 stimulated damage by radioresistant immune cells. The second caveat, which may not be so easily overcome, is that nonhematopoietic cell types which do not express IFN λ R, such as pulmonary endothelial cells, could be a potent source of proinflammatory cytokines (Sommerreyns et al., 2008). Teijaro et al. have proposed that endothelial cells are an potent source of IFN α β -driven proinflammatory cytokines in IAV infection (Teijaro et al., 2011). If this scenario is true, then IFN α 4 treatment could still augment pulmonary inflammation through stimulation of endothelial cells and thereby induce pathology. It will be of great interest to see the outcome of this experiment.

Although we characterised IFN λ treatment as protective during IAV infection, this was demonstrated in low IFN $\alpha\beta$ responding hosts. We therefore have no indication if this will translate into a genetic background with a predisposition for high IFN $\alpha\beta$ responsiveness. In a high IFN $\alpha\beta$ setting, IFN λ mediated control of IAV replication may not be sufficient to protect the host from IFN $\alpha\beta$ mediated tissue damage and therefore, severe disease. AEC death was particularly observable late in infection, in spite of viral loads being very low in 129 mice and undetectable in B6 mice. Therefore, complementing IFN λ treatment with blockade of IFN $\alpha\beta$ signalling late in IAV infection may ensure control of IAV replication and protection from lung tissue damage. Of course this would have to be investigated intensively to ensure blockade of IFN $\alpha\beta$ R did not promote disease by hindering the IAV specific adaptive immune response or alveolar macrophage clearance of cellular debris. Moreover, IFN $\alpha\beta$ R antagonism late in infection would only be protective if IFN $\alpha\beta$ induction of AEC apoptosis is a result of IFN $\alpha\beta$ acting on specific cell types present in the lung late in infection and not a consequence of the overall duration of IFN $\alpha\beta$ signalling in the lung. If duration of IFN $\alpha\beta$ signal or high concentrations of IFN $\alpha\beta$ early in infection are upstream of AEC apoptosis then the timing or frequency of IFN $\alpha\beta$ R blockade would have to be adjusted. Alternatively, perhaps lowering IFN α concentrations, through pDC depletion, rather than entirely ablating IFN $\alpha\beta$ R signalling, would be a better complement to IFN λ treatment in high responders.

As discussed in Chapter 4, the redundancy between IFN $\alpha\beta$ and IFN λ in viral infection is directly dependent upon virus tissue tropism and as mentioned, this concept was nicely demonstrated by comparison of IFN $\alpha\beta$ R $^{-/-}$ and IFN λ R $^{-/-}$ mice infected with human reovirus type 3 and assessment of virally infected cells in the

gut. In the gut, IFN $\alpha\beta$ R and IFN λ R expression on different cell types is mutually exclusive and therefore, the pattern of viral infection was contingent on cell types being unable to respond to IFN signalling due to receptor deficiency (Mahlakoiv et al., 2015). In contrast, lung AECs can respond to both IFN $\alpha\beta$ and IFN λ and productive replication to IAV is generally restricted to AECs. However, some strains of IAV, particularly highly pathogenic avian strains, have been shown to possess polybasic cleavage sites on their HA molecules and thus are not dependent upon proteases only expressed by AECs for maturation of the virion (Stieneke-Grober et al., 1992). Significantly, under these conditions, exogenous IFN λ treatment may not elicit protective effects. Instead it may even increase immunopathology by driving IAV to replicate in immune cells and thereby increasing the availability of viral PAMPs in cell types programmed to generate large quantities of proinflammatory cytokines. In sharp contrast to possible treatment regimes already discussed in this section, exogenous IFN λ treatment supplemented with exogenous IFN $\alpha\beta$ may be beneficial under these circumstances.

We utilise the restricted expression of IFN λ R to induce an antiviral state in cells vulnerable to IAV infection without perturbing the immune response, however one can assume that we were merely capitalising on evolution's design. An IFN that specifically acts on cells that have an elevated risk of pathogen exposure allows for these cells to autonomously protect themselves from invading pathogens. It is therefore likely that many of the pathogens humans are exposed to in daily life are dealt with by local low-grade IFN λ responses with little to no recruitment of immune cells. IFN λ can be thought of as an autonomous virus defence system of epithelial barriers that may have evolved to avoid unnecessarily

frequent triggering of the IFN $\alpha\beta$ system which can lead to exacerbated inflammation. Over half a century has past since the discovery of IFN $\alpha\beta$ and in this time much research has demonstrated a vital role for this IFN in defence against many viruses. However, to study the effects of IFN $\alpha\beta$ in viral infection, a model must be used where infection can be established. For instance, to study systemic viral infection, a virus must be injected, thereby bypassing protective innate host defence mechanisms such as mucus or IFN λ on epithelial cell surfaces. Systemic viral infection involves viruses that can infect and replicate in multiple cell types, particularly in immune cells and in these cells IFN λ has limited or no ability to induce ISG expression, consequently IFN $\alpha\beta$ has unique roles and is absolutely required for protection. However, one must wonder how often viruses overcome innate immunity on mucosal surfaces and achieve systemic infection in nature? SNPs that decrease or ablate the shared signalling cascade downstream of IFN $\alpha\beta$ R and IFN λ R result in acute sensitivity to viral infections and this is often attributed to loss of protection mediated by IFN $\alpha\beta$, while IFN λ is overlooked. However, Duncan et al. recently identified an individual with a homozygous mutation in the high-affinity chain of the IFN $\alpha\beta$ R (IFNAR2) that rendered cells unresponsive to IFN $\alpha\beta$. This individual came to notice after succumbing to encephalitis induced by inoculation of the live attenuated measles, mumps, and rubella (MMR) vaccine. However, until vaccination this individual had not shown any indication of heightened susceptibility to viral pathogens (Duncan et al., 2015). It appears that IFN λ and other host defence mechanisms at surfaces exposed to pathogens were sufficient to ensure host protection from viral induced disease, and it could be argued that the defect in IFN $\alpha\beta$ R signalling may not have been detected if the individual had not been directly challenged with live, albeit attenuated virus by a

route which circumvented these surface defences. To date, the protective role of IFN $\alpha\beta$ in clinical disease may therefore be over estimated and the importance of IFN λ under appreciated.

It is of interest to assess the clinical importance of host intrinsic elevated IFN $\alpha\beta$ responsiveness. Unlike experimental mice that live in specific pathogen-free conditions, humans are under constant polymicrobial exposure. Could this IFN $\alpha\beta$ elevated response phenotype still exist in the context of the constant immunostimulation and if so, would it still manifest as pathogenic? It is possible that propensity to make high concentrations of IFN $\alpha\beta$ (or IFN λ , as they appear to go hand in hand as evidenced in 129 mice) in response to low levels of stimulus may in fact make high IFN $\alpha\beta$ responsive hosts more resistant to infection, by inducing a heightened or prolonged antiviral state before novel exposure to a subsequent virus, much like in the pretreatment regimen in Chapter 4. Indeed, two studies have demonstrated that commensal bacteria provide tonic immune stimulation that establishes a basal level of activation in the innate immune system and this is required for optimal antiviral immunity. Treatment of mice with broad-spectrum antibiotic regimes prior to IAV infection lowers this tonic signal and thereby impaired induction of IFN $\alpha\beta$ consequently increasing IAV titres in the lung and disease burden (Abt et al., 2012; Ichinohe et al., 2011). Increased immunostimulation in humans could intensify this IFN $\alpha\beta$ responsiveness and this could be positive or negative for the host. A high IFN $\alpha\beta$ responsive phenotype may result in higher baseline expression of ISG therefore providing a better guard against virus invasion or a stronger IFN $\alpha\beta$ response early in infection may shut down viral replication before it is established. Of course this does not preclude high IFN $\alpha\beta$ responding phenotype from presenting with severe disease if an IAV

infection is established, as was observed in 129 mice and possibly individuals with DS.

Dp8Tyb mice are trisomic for a short section of murine chromosome 16 containing the IFNAR1 gene, we employed this strain of mouse to test whether or not increasing gene dosage of IFNAR1 could increase IFN $\alpha\beta$ mediated immunopathology in IAV infection. Although preliminary, results attained in Dp8Tyb mice remarkably parallel disease features observed in 129 mice. Of course further characterisation of this phenotype is required in particular, assessment of the proinflammatory cytokine milieu and frequency of AEC death induced by IAV infection in this strain compared to their littermate controls. Moreover, a better understanding of viral control and induction of IAV specific adaptive immune responses by Dp8Tyb mice would allow us to firstly, understand if the trisomy possessed by Dp8Tyb mice affects these aspects of the immune response to IAV and if so is this more important than immune mediated tissue damage in driving disease severity. If, as we hypothesise IFNAR1 triplication is augmenting IFN $\alpha\beta$ R signalling in Dp8Tyb mouse many of the treatments discussed in this chapter such as depletion of pDCs or blockade of TLR7 or IFN $\alpha\beta$ R signalling could be applied to this model. Most interesting, would be to demonstrate a proof of principle by breeding Dp8Tyb mice with IFN $\alpha\beta$ R $^{+/-}$ (B6) mice, thereby generating offspring which are trisomic for the entire duplicated region of Dp8Tyb with the exception of IFNAR1 (along with appropriate littermate controls). Finally, correlation of severe disease markers identified in this model with IAV infected DS patients would demonstrate the relevance of this model and as this is a genetically defined population, may lead to the design of IFN $\alpha\beta$ dampening therapies to treat severe IAV in DS.

Data presented in this thesis have shed light on the pathogenic potential of IFN $\alpha\beta$ in IAV infection. We have identified IFN $\alpha\beta$ to be a candidate factor upstream of the cytokine storm in humans and also delineated an IFN $\alpha\beta$ dependent pathway that leads to AEC death during IAV infection. This work also allows us to recognise the somewhat opposing actions of IFN $\alpha\beta$ in IAV infection: the cell intrinsic antiviral response and the cell extrinsic immunomodulatory effects. Comparison with IFN λ allowed for the disentanglement of these effects and has revealed IFN λ as a potential treatment for IAV induced disease. Finally, the definition of features specific to the IFN $\alpha\beta$ driven pathogenic response to IAV will allow for future investigation of upstream genetic elements that lead to this elevated IFN $\alpha\beta$ responsive phenotype, with hopes for translation into human studies.

Acknowledgements

It's quite late, or particularly early, depending on your opinion of what hours a person should keep. It is also quite late in terms of my thesis, I am waiting for the next set of edits from Andreas which will hopefully be the last, yet it may be still too early to write my Acknowledgements as once the text is complete I will begin the risky business of formatting. Anyway, here we are regardless, at 3.06am on a Tuesday morning. They say that the Acknowledgments are the only thing people read of your thesis once you've passed your viva so I guess I'd better make them good. I should also say they're also going to be long, because let's be frank, writing this thesis has destroyed my looks so I'm never going to get that Oscar.

Where to begin? Well, there are two sets of shoulders that my extreme gratitude falls upon. Without these two people I would not have survived my PhD and even if I had, I would never have come out as such a happy and well-rounded scientist. These are of course, my long-suffering fiancé Kye Manefield, and my supervisor and friend, Dr. Andreas Wack (who surely suffered less than Kye over the last 5 years). On these two sets of shoulders I leant on, cried on and stood upon to achieve all that I have over the last 5 years, I cannot communicate how truly grateful I am.

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I have made so many dear friends here, I think the first of which (aside from the Wack lab of course) was the lovely Joao Durate, I miss having you around, if nothing else then for new book recommendations. You introduced me to the social side of NIMR and for that my heart thanks you... although my liver is less grateful. Also from the Stockinger lab I'd like to thank Matteo, you have a special way about you that always makes me smile and I'm also happy I had someone to share the suffering of Open University regulations with.

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for the constant verbal battering you receive from me, generally you don't deserve it... generally. The point is, I appreciate your friendship and think you're funny and not even that far underneath it all, you have a good heart, thank you for checking up on me over and over in these final days and being the Bob to my Charlotte.

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I could go on and on about NIMR, it's an amazing place to work and I am incredibly sad that it's ending. I hope the NIMR spirit transfers to the Crick when the institute eventually moves to Kings Cross. The science and the people make this place so unique and precious and although I'm leaving I'll always hold NIMR in my heart and every new place I go I'll try to sow a little seed of it to carry on the legacy.

Finally, I want to thank the people in my life that I'm terrible at keeping in contact with, you have helped make me who I am (take that as you will). Thank you to my Tooting Family, Nat, Rowe, Bree, Kirsty, Nury, Mick and Adam who, as the name implies was my family for the time we lived in Tooting. Christmas time with you all was a sight to behold. Thank you to Dr. Simon Phipps, Mr. Woods and Geoffrey Coady who excited my love of science and encouraged me along the way. To my lovely cousins Joel and Katy, I don't even mind that you keep having weddings on my birthday. To Tim Gardner whose haughty backhanded compliments do me more good than he undoubtedly intends. To my far off sisters, Naomi, Rach, Lina and Jess your friendship has saw me through the worst time of my life, I'll always be grateful.

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Oh God, that's enough. I hope I remembered everyone. I guess there's nothing else left but to thank you dear reader... 8 pages of rambling acknowledgements is a lot of read through, but also I guess I should wish you luck, because likely you are Teresa starting to write your own thesis, then perhaps Helena, later Milena and then who knows...

If you are reading this on the cusp of writing up, good luck and you can do it, it'll seem unlikely at times but I've seen you and I know you can.

Appendix

Table 1: FACS Antibodies.

Antigen	Fluorochrome Conjugate	Company
120G8	FITC	Biolegend
B220	PerCP Cy5.5	Biolegend
B220	APC Cy7	Biolegend
CD11b	PE Cy7	Biolegend
CD11b	BV711	Biolegend
CD11c	Pacific Blue	Biolegend
CD11c	PerCP Cy5.5	Biolegend
CD11c	BV605	Biolegend
CD138	PE	Biolegend
CD18	PE Cy7	Biolegend
CD19	BV650	Biolegend
CD23	PE	Biolegend
CD38	PE Cy5	Biolegend
CD38	PE Cy7	Biolegend
CD3e	APC Cy7	Biolegend
CD3e	AF700	Biolegend
CD3e	APC	Biolegend
CD4	APC	Biolegend
CD4	PE Cy7	Biolegend
CD4	BV605	Biolegend
CD44	PE	Biolegend
CD45	APC	Biolegend
CD45.1	APC Cy7	Biolegend
CD45.2	Pacific Blue	Biolegend
CD45.2	BV650	Biolegend
CD69	FITC	Biolegend
CD69	PE Cy7	Biolegend
CD8a	PerCP Cy5.5	Biolegend

Antigen	Fluorochrome Conjugate	Company
CD8a	BV785	Biolegend
CD86	FITC	Biolegend
CD93	APC	Biolegend
DR5	PE	Biolegend
DX5	Pacific Blue	Biolegend
Ecadtherin	FITC	Biolegend
Ep CAM	APC Cy7	Biolegend
F4/80	APC	Biolegend
Fas	PE	Biolegend
GL7	eF660	Biolegend
IFNAR1	PE	Biolegend
IgD	Pacific Blue	Biolegend
IgM	FITC	Biolegend
Ly6C	PerCP Cy5.5	Biolegend
Ly6C	FITC	Biolegend
Ly6G	APC-CY7	Biolegend
Ly6G	AF700	Biolegend
Ly6G	FITC	Biolegend
MHCII	APC-CY7	Biolegend
NK1.1	PE-Cy7	Biolegend
NKp46	FITC	Biolegend
NKp46	PE	Biolegend
		Vector Laboratories
PNA	FITC	
Sca-1	Pacific Blue	Biolegend
Siglec F	---	Miltenyi
Siglec H	PE	Biolegend
TRAIL	PE	Biolegend
Streptavidin	PE Dazzle	Biolegend

Table 2: Gene List, Figure 30, Genes upregulated by both IFN λ 2 and IFN α 4 treatment.

Symbol	Fold change([IFNL] vs [mock])	Fold change([IFNa4] vs [mock])
1200013B22Rik	1.3181694	1.5204556
1500012F01Rik	1.1593927	1.5565454
2010005H15Rik	1.5644674	1.4659208
A130072J07	1.4594407	1.5547892
AI481105	1.2891465	1.5229447
Akp2	1.0820922	1.5346539
Apobec1	1.0573468	1.5541328
Atf3	1.3182873	1.5506775
Atp10a	1.1806552	1.5575788
Axud1	1.2855201	1.522764
Azi2	1.1990424	1.5377803
B2m	1.4298825	1.5584313
B630009I04Rik	1.16654	1.5465243
BC013672	1.2780781	1.5458938
BC024561	1.1399192	1.5717908
BC049354	1.1393989	1.548339
C4	1.0964634	1.5194741
C430002D13Rik	1.5289665	1.3244027
Cd180	1.0180258	1.5485319
Clecsf12	1.2993085	1.54169
Ctps	1.3228042	1.5746181
Ctps	1.261551	1.5738385
Ctsb	1.2983029	1.5082626
Ctsz	1.1812767	1.5360832
Cxcl14	1.0903791	1.5670463
Cyr61	1.659218	1.1214038
Cyr61	1.5165956	1.1201091
Ddx24	1.2157705	1.5293789
Dusp1	1.8283868	1.0965629
Enpp4	1.2865506	1.5081517
F630107D10Rik	1.0873708	1.5366734
Fos	2.5179932	1.4653891
Gadd45g	1.1974773	1.5161738
Gsdmdc1	1.1996303	1.5237099
H2-Q5	1.2226307	1.5184234
Hspb6	1.0921898	1.5537612
Ifi203	1.048598	1.5026933
Irf1	1.0727218	1.5019846
Irf5	-1.0438304	1.5022235

Table 2 cont: Gene List, Figure 30, Genes upregulated by both IFN λ 2 and IFN α 4 treatment.

Symbol	Fold change([IFNL] vs [mock])	Fold change([IFNa4] vs [mock])
Irgm	1.1785004	1.5476046
Itih4	1.3737946	1.5188125
Klra7	1.0613177	1.508586
Klre1	1.0261391	1.5026431
LOC381010	1.2453744	1.5319322
Mill2	1.2839385	1.5445967
Mthfd2	1.1744696	1.5579389
Pbef1	1.3799819	1.5217743
Pla1a	1.1087766	1.5226134
Plod3	1.2406632	1.5209503
Ppp1r14d	1.4666433	1.5208132
Prkcdbp	1.1568284	1.5361063
Rarres2	1.1477493	1.5292376
Rhoc	1.0478281	1.5307486
scl0002116.1_6	1.2034056	1.5256225
Tacstd2	1.6001923	1.3650656
Tgfb1	1.3123162	1.5621724
Tgfb1	1.2293031	1.5578811
Thbs1	1.3436913	1.5134673
Tinagl	1.0367861	1.570407
Tinagl	-1.0254425	1.5228773
Tlr7	1.0034118	1.5203454
Tmem176a	1.0172911	1.506547
Tmsb10	-1.0172322	1.5313413
Upp1	1.0511423	1.5409486
Xcl1	-1.0007374	1.5644635

Table 3: Gene List, Figure 30, IFN α 4 specific genes.

Symbol	Fold change([IFNL] vs [mock])	Fold change([IFNa4] vs [mock])
2210415K03Rik	3.1301882	1.0827683
A630077B13Rik	2.3969235	1.0720068
Aif1	2.2063124	1.1540902
BC049975	3.3051805	1.2625252
C2	3.360701	1.1232857
Ccl12	2.309329	1.205247
Ccl12	2.7600749	1.3374633
Ccl4	3.5691519	1.0817877
Ccl4	2.6236436	1.0771163
Ccl7	3.2992876	1.1421381
Ccl8	3.0029647	1.2521714
Ccr5	2.2546341	1.1044458
Ccr5	2.258544	1.1143029
Cxcl1	2.3235338	1.282936
Cxcl13	3.7380526	1.2582576
Daxx	2.6530592	1.3208724
Fcgr1	2.4304502	1.1923883
Fcrl3	2.3719475	1.0939442
Gbp2	3.9855018	1.2847389
Gbp2	3.6796377	1.2362367
Gvin1	2.667871	1.1066488
Ifi205	2.655555	1.1526281
Ifi205	3.6843872	1.3771138
IL1RA	2.1915321	1.1259896
Irg1	2.4912689	1.2694376
LOC226690	3.2811322	1.1577247
LOC226691	3.490336	1.3775985
LOC381276	2.4568229	1.2585015
LOC626578	3.1149218	1.2755919
Ms4a6d	3.8090258	1.3229121
Nrap	2.496111	1.2824109
Orm1	2.3607984	1.2848341
Orm2	2.1883066	1.0642031
Pla1a	2.583456	1.17412
Plac8	2.8865275	1.1678452
scl000868.1_2	2.5899966	1.1220796
Sfrp1	2.6343455	1.094101
Sn	2.4187682	-1.0040914

Bibliography

- Abt, M.C., L.C. Osborne, L.A. Monticelli, T.A. Doering, T. Alenghat, G.F. Sonnenberg, M.A. Paley, M. Antenus, K.L. Williams, J. Erikson, E.J. Wherry, and D. Artis. 2012. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37:158-170.
- Ada, G.L., and P.D. Jones. 1986. The immune response to influenza infection. *Current topics in microbiology and immunology* 128:1-54.
- Agrati, C., C. Gioia, E. Lalle, E. Cimini, C. Castilletti, O. Armignacco, F.N. Lauria, F. Ferraro, M. Antonini, G. Ippolito, M.R. Capobianchi, and F. Martini. 2010. Association of profoundly impaired immune competence in H1N1v-infected patients with a severe or fatal clinical course. *The Journal of infectious diseases* 202:681-689.
- Akaike, T., Y. Noguchi, S. Ijiri, K. Setoguchi, M. Suga, Y.M. Zheng, B. Dietzschold, and H. Maeda. 1996. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proceedings of the National Academy of Sciences of the United States of America* 93:2448-2453.
- Alberts, R., B. Srivastava, H. Wu, N. Viegas, R. Geffers, F. Klawonn, N. Novoselova, T.Z. do Valle, J.J. Panthier, and K. Schughart. 2010. Gene expression changes in the host response between resistant and susceptible inbred mouse strains after influenza A infection. *Microbes and infection / Institut Pasteur* 12:309-318.
- Albright, F.S., P. Orlando, A.T. Pavia, G.G. Jackson, and L.A. Cannon Albright. 2008. Evidence for a heritable predisposition to death due to influenza. *The Journal of infectious diseases* 197:18-24.
- Alcais, A., L. Quintana-Murci, D.S. Thaler, E. Schurr, L. Abel, and J.L. Casanova. 2010. Life-threatening infectious diseases of childhood: single-gene inborn errors of immunity? *Annals of the New York Academy of Sciences* 1214:18-33.
- Aldridge, J.R., Jr., C.E. Moseley, D.A. Boltz, N.J. Negovetich, C. Reynolds, J. Franks, S.A. Brown, P.C. Doherty, R.G. Webster, and P.G. Thomas. 2009. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 106:5306-5311.
- Allen, I.C., M.A. Scull, C.B. Moore, E.K. Holl, E. McElvania-TeKippe, D.J. Taxman, E.H. Guthrie, R.J. Pickles, and J.P. Ting. 2009. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30:556-565.
- Ank, N., M.B. Iversen, C. Bartholdy, P. Staeheli, R. Hartmann, U.B. Jensen, F. Dagnaes-Hansen, A.R. Thomsen, Z. Chen, H. Haugen, K. Klucher, and S.R. Paludan. 2008. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *Journal of immunology* 180:2474-2485.
- Ank, N., H. West, C. Bartholdy, K. Eriksson, A.R. Thomsen, and S.R. Paludan. 2006a. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *Journal of virology* 80:4501-4509.
- Ank, N., H. West, and S.R. Paludan. 2006b. IFN-lambda: novel antiviral cytokines. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 26:373-379.
- Antonopoulou, A., F. Baziaka, T. Tsaganos, M. Raftogiannis, P. Koutoukas, A. Spyridaki, M. Mouktaroudi, A. Kotsaki, A. Savva, M. Georgitsi, and E.J.

- Giamarellos-Bourboulis. 2012. Role of tumor necrosis factor gene single nucleotide polymorphisms in the natural course of 2009 influenza A H1N1 virus infection. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* 16:e204-208.
- Arankalle, V.A., K.S. Lole, R.P. Arya, A.S. Tripathy, A.Y. Ramdasi, M.S. Chadha, S.A. Sangle, and D.B. Kadam. 2010. Role of host immune response and viral load in the differential outcome of pandemic H1N1 (2009) influenza virus infection in Indian patients. *PloS one* 5:
- Asmuth, D.M., R.L. Murphy, S.L. Rosenkranz, J.J. Lertora, S. Kottlil, Y. Cramer, E.S. Chan, R.T. Schooley, C.R. Rinaldo, N. Thielman, X.D. Li, S.M. Wahl, J. Shore, J. Janik, R.A. Lempicki, Y. Simpson, R.B. Pollard, and A.C.T.G.A. Team. 2010. Safety, tolerability, and mechanisms of antiretroviral activity of pegylated interferon Alfa-2a in HIV-1-monoinfected participants: a phase II clinical trial. *The Journal of infectious diseases* 201:1686-1696.
- Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature immunology* 2:1144-1150.
- Asselin-Paturel, C., G. Brizard, J.J. Pin, F. Briere, and G. Trinchieri. 2003. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *Journal of immunology* 171:6466-6477.
- Bahl, K., A. Huebner, R.J. Davis, and R.M. Welsh. 2010. Analysis of apoptosis of memory T cells and dendritic cells during the early stages of viral infection or exposure to toll-like receptor agonists. *Journal of virology* 84:4866-4877.
- Balzarolo, M., J.J. Karrich, S. Engels, B. Blom, J.P. Medema, and M.C. Wolkers. 2012. The transcriptional regulator NAB2 reveals a two-step induction of TRAIL in activated plasmacytoid DCs. *European journal of immunology* 42:3019-3027.
- Barchet, W., M. Cella, B. Odermatt, C. Asselin-Paturel, M. Colonna, and U. Kalinke. 2002. Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. *The Journal of experimental medicine* 195:507-516.
- Barro-Soria, R., S. Rebolledo, S.I. Liin, M.E. Perez, K.J. Sampson, R.S. Kass, and H.P. Larsson. 2014. KCNE1 divides the voltage sensor movement in KCNQ1/KCNE1 channels into two steps. *Nature communications* 5:3750.
- Baskin, C.R., H. Bielefeldt-Ohmann, T.M. Tumpey, P.J. Sabourin, J.P. Long, A. Garcia-Sastre, A.E. Tolnay, R. Albrecht, J.A. Pyles, P.H. Olson, L.D. Aicher, E.R. Rosenzweig, K. Murali-Krishna, E.A. Clark, M.S. Kotur, J.L. Fornek, S. Proll, R.E. Palermo, C.L. Sabourin, and M.G. Katze. 2009. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* 106:3455-3460.
- Baum, A., R. Sachidanandam, and A. Garcia-Sastre. 2010. Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. *Proceedings of the National Academy of Sciences of the United States of America* 107:16303-16308.

- Beigel, J.H., J. Farrar, A.M. Han, F.G. Hayden, R. Hyer, M.D. de Jong, S. Lochindarat, T.K. Nguyen, T.H. Nguyen, T.H. Tran, A. Nicoll, S. Touch, K.Y. Yuen, and A.H. Writing Committee of the World Health Organization Consultation on Human Influenza. 2005. Avian influenza A (H5N1) infection in humans. *The New England journal of medicine* 353:1374-1385.
- Beilharz, M.W., J.M. Cummins, and A.L. Bennett. 2007. Protection from lethal influenza virus challenge by oral type 1 interferon. *Biochemical and biophysical research communications* 355:740-744.
- Belisle, S.E., J.R. Tisoncik, M.J. Korth, V.S. Carter, S.C. Proll, D.E. Swayne, M. Pantin-Jackwood, T.M. Tumpey, and M.G. Katze. 2010. Genomic profiling of tumor necrosis factor alpha (TNF-alpha) receptor and interleukin-1 receptor knockout mice reveals a link between TNF-alpha signaling and increased severity of 1918 pandemic influenza virus infection. *Journal of virology* 84:12576-12588.
- Belz, G.T., P.G. Stevenson, and P.C. Doherty. 2000. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. *Journal of immunology* 165:2404-2409.
- Benedict, C.A., and C.F. Ware. 2012. TRAIL: not just for tumors anymore? *The Journal of experimental medicine* 209:1903-1906.
- Bergmann, M., A. Garcia-Sastre, E. Carnero, H. Pehamberger, K. Wolff, P. Palese, and T. Muster. 2000. Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *Journal of virology* 74:6203-6206.
- Berkhoff, E.G., M.M. Geelhoed-Mieras, R.A. Fouchier, A.D. Osterhaus, and G.F. Rimmelzwaan. 2007. Assessment of the extent of variation in influenza A virus cytotoxic T-lymphocyte epitopes by using virus-specific CD8+ T-cell clones. *The Journal of general virology* 88:530-535.
- Bernardo, A.R., J.M. Cosgaya, A. Aranda, and A.M. Jimenez-Lara. 2013. Synergy between RA and TLR3 promotes type I IFN-dependent apoptosis through upregulation of TRAIL pathway in breast cancer cells. *Cell death & disease* 4:e479.
- Bhattacharya, S., K.V. Katlinski, M. Reichert, S. Takano, A. Brice, B. Zhao, Q. Yu, H. Zheng, C.J. Carbone, Y.V. Katlinskaya, N.A. Leu, K.A. McCorkell, S. Srinivasan, M. Gironde, H. Rui, M.J. May, N.G. Avadhani, A.K. Rustgi, and S.Y. Fuchs. 2014. Triggering ubiquitination of IFNAR1 protects tissues from inflammatory injury. *EMBO molecular medicine* 6:384-397.
- Biddison, W.E., and S. Shaw. 1979. Differences in HLA antigen recognition by human influenza virus-immune cytotoxic T cells. *Journal of immunology* 122:1705-1709.
- Bjorck, P. 2001. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* 98:3520-3526.
- Blackwell, J.M., S.E. Jamieson, and D. Burgner. 2009. HLA and infectious diseases. *Clinical microbiology reviews* 22:370-385, Table of Contents.
- Blazek, K., H.L. Eames, M. Weiss, A.J. Byrne, D. Perocheau, J.E. Pease, S. Doyle, F. McCann, R.O. Williams, and I.A. Udalova. 2015. IFN-lambda resolves inflammation via suppression of neutrophil infiltration and IL-1beta production. *The Journal of experimental medicine* 212:845-853.
- Bloemers, B.L., L. Bont, R.A. de Weger, S.A. Otto, J.A. Borghans, and K. Tesselaar. 2011. Decreased thymic output accounts for decreased naive T cell

numbers in children with Down syndrome. *Journal of immunology* 186:4500-4507.

- Bloemers, B.L., A.M. van Furth, M.E. Weijerman, R.J. Gemke, C.J. Broers, K. van den Ende, J.L. Kimpen, J.L. Strengers, and L.J. Bont. 2007. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. *Pediatrics* 120:e1076-1081.
- Bodmer, J.L., P. Meier, J. Tschopp, and P. Schneider. 2000. Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. *The Journal of biological chemistry* 275:20632-20637.
- Boon, A.C., G. de Mutsert, Y.M. Graus, R.A. Fouchier, K. Sintnicolaas, A.D. Osterhaus, and G.F. Rimmelzwaan. 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype. *Journal of virology* 76:582-590.
- Boon, A.C., G. de Mutsert, D. van Baarle, D.J. Smith, A.S. Lapedes, R.A. Fouchier, K. Sintnicolaas, A.D. Osterhaus, and G.F. Rimmelzwaan. 2004. Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. *Journal of immunology* 172:2453-2460.
- Boon, A.C., J. deBeauchamp, A. Hollmann, J. Luke, M. Kotb, S. Rowe, D. Finkelstein, G. Neale, L. Lu, R.W. Williams, and R.J. Webby. 2009. Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. *Journal of virology* 83:10417-10426.
- Boon, A.C., D. Finkelstein, M. Zheng, G. Liao, J. Allard, K. Klumpp, R. Webster, G. Peltz, and R.J. Webby. 2011. H5N1 influenza virus pathogenesis in genetically diverse mice is mediated at the level of viral load. *mBio* 2:
- Bottcher-Friebertshauser, E., C. Freuer, F. Sielaff, S. Schmidt, M. Eickmann, J. Uhlenhorff, T. Steinmetzer, H.D. Klenk, and W. Garten. 2010. Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. *Journal of virology* 84:5605-5614.
- Bouvier, N.M., and A.C. Lowen. 2010. Animal Models for Influenza Virus Pathogenesis and Transmission. *Viruses* 2:1530-1563.
- Bouvier, N.M., and P. Palese. 2008. The biology of influenza viruses. *Vaccine* 26 Suppl 4:D49-53.
- Brandenburg, B., W. Koudstaal, J. Goudsmit, V. Klaren, C. Tang, M.V. Bujny, H.J. Korse, T. Kwaks, J.J. Otterstrom, J. Juraszek, A.M. van Oijen, R. Vogels, and R.H. Friesen. 2013. Mechanisms of hemagglutinin targeted influenza virus neutralization. *PloS one* 8:e80034.
- Brincks, E.L., P. Gurung, R.A. Langlois, E.A. Hemann, K.L. Legge, and T.S. Griffith. 2011. The magnitude of the T cell response to a clinically significant dose of influenza virus is regulated by TRAIL. *Journal of immunology* 187:4581-4588.
- Brincks, E.L., A. Katewa, T.A. Kucaba, T.S. Griffith, and K.L. Legge. 2008a. CD8 T cells utilize TRAIL to control influenza virus infection. *Journal of immunology* 181:4918-4925.
- Brincks, E.L., T.A. Kucaba, K.L. Legge, and T.S. Griffith. 2008b. Influenza-induced expression of functional tumor necrosis factor-related apoptosis-inducing ligand on human peripheral blood mononuclear cells. *Human immunology* 69:634-646.

- Broers, C.J., R.J. Gemke, M.E. Weijerman, K.F. van der Sluijs, and A.M. van Furth. 2012. Increased pro-inflammatory cytokine production in Down Syndrome children upon stimulation with live influenza A virus. *Journal of clinical immunology* 32:323-329.
- Bruijn, M., L.B. van der Aa, R.R. van Rijn, A.P. Bos, and J.B. van Woensel. 2007. High incidence of acute lung injury in children with Down syndrome. *Intensive care medicine* 33:2179-2182.
- Burgio, G.R., A. Lanzavecchia, R. Maccario, A. Vitiello, A. Plebani, and A.G. Ugazio. 1978. Immunodeficiency in Down's syndrome: T-lymphocyte subset imbalance in trisomic children. *Clinical and experimental immunology* 33:298-301.
- Campbell, D.J., and M.A. Koch. 2011. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nature reviews. Immunology* 11:119-130.
- Carragher, D.M., D.A. Kaminski, A. Moquin, L. Hartson, and T.D. Randall. 2008. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *Journal of immunology* 181:4168-4176.
- Carsetti, R., D. Valentini, V. Marcellini, M. Scarsella, E. Marasco, F. Giustini, A. Bartuli, A. Villani, and A.G. Ugazio. 2015. Reduced numbers of switched memory B cells with high terminal differentiation potential in Down syndrome. *European journal of immunology* 45:903-914.
- Casanova, J.L., S.M. Holland, and L.D. Notarangelo. 2012. Inborn errors of human JAKs and STATs. *Immunity* 36:515-528.
- Cate, T.R. 1987. Clinical manifestations and consequences of influenza. *The American journal of medicine* 82:15-19.
- Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nature medicine* 5:919-923.
- Cervantes-Barragan, L., K.L. Lewis, S. Firner, V. Thiel, S. Hugues, W. Reith, B. Ludewig, and B. Reizis. 2012. Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 109:3012-3017.
- Cervantes-Barragan, L., R. Züst, F. Weber, M. Spiegel, K.S. Lang, S. Akira, V. Thiel, and B. Ludewig. 2007. Control of coronavirus infection through plasmacytoid dendritic-cell-derived type I interferon. *Blood* 109:1131-1137.
- Chang, E.Y., B. Guo, S.E. Doyle, and G. Cheng. 2007a. Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. *Journal of immunology* 178:6705-6709.
- Chang, W.L., E.S. Coro, F.C. Rau, Y. Xiao, D.J. Erle, and N. Baumgarth. 2007b. Influenza virus infection causes global respiratory tract B cell response modulation via innate immune signals. *Journal of immunology* 178:1457-1467.
- Chaperot, L., A. Blum, O. Manches, G. Lui, J. Angel, J.P. Molens, and J. Plumas. 2006. Virus or TLR agonists induce TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. *Journal of immunology* 176:248-255.
- Chehimi, J., S.E. Starr, H. Kawashima, D.S. Miller, G. Trinchieri, B. Perussia, and S. Bandyopadhyay. 1989. Dendritic cells and IFN-alpha-producing cells are

- two functionally distinct non-B, non-monocytic HLA-DR⁺ cell subsets in human peripheral blood. *Immunology* 68:486-490.
- Chen, H., M.A. Morris, C. Rossier, J.L. Blouin, and S.E. Antonarakis. 1995. Cloning of the cDNA for the human ATP synthase OSCP subunit (ATP50) by exon trapping and mapping to chromosome 21q22.1-q22.2. *Genomics* 28:470-476.
- Chen, Y.L., T.T. Chen, L.M. Pai, J. Wesoly, H.A. Bluysen, and C.K. Lee. 2013. A type I IFN-Flt3 ligand axis augments plasmacytoid dendritic cell development from common lymphoid progenitors. *The Journal of experimental medicine* 210:2515-2522.
- Cheung, C.Y., L.L. Poon, A.S. Lau, W. Luk, Y.L. Lau, K.F. Shortridge, S. Gordon, Y. Guan, and J.S. Peiris. 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* 360:1831-1837.
- Ciancanelli, M.J., S.X. Huang, P. Luthra, H. Garner, Y. Itan, S. Volpi, F.G. Lafaille, C. Trouillet, M. Schmolke, R.A. Albrecht, E. Israelsson, H.K. Lim, M. Casadio, T. Hermesh, L. Lorenzo, L.W. Leung, V. Pedernana, B. Boisson, S. Okada, C. Picard, B. Ringuier, F. Troussier, D. Chaussabel, L. Abel, I. Pellier, L.D. Notarangelo, A. Garcia-Sastre, C.F. Basler, F. Geissmann, S.Y. Zhang, H.W. Snoeck, and J.L. Casanova. 2015. Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency. *Science* 348:448-453.
- Cilloniz, C., M.J. Pantin-Jackwood, C. Ni, V.S. Carter, M.J. Korth, D.E. Swayne, T.M. Tumpey, and M.G. Katze. 2012. Molecular signatures associated with Mx1-mediated resistance to highly pathogenic influenza virus infection: mechanisms of survival. *Journal of virology* 86:2437-2446.
- Clements, M.L., and B.R. Murphy. 1986. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *Journal of clinical microbiology* 23:66-72.
- Cook, D.N., M.A. Beck, T.M. Coffman, S.L. Kirby, J.F. Sheridan, I.B. Pragnell, and O. Smithies. 1995. Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* 269:1583-1585.
- Coro, E.S., W.L. Chang, and N. Baumgarth. 2006. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. *Journal of immunology* 176:4343-4351.
- Costa-Carvalho, B.T., R.M. Martinez, A.T. Dias, C.A. Kubo, P. Barros-Nunes, L. Leiva, D. Sole, M.M. Carneiro-Sampaio, C.K. Naspitz, and R.U. Sorensen. 2006. Antibody response to pneumococcal capsular polysaccharide vaccine in Down syndrome patients. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.]* 39:1587-1592.
- Coulombe, F., J. Jaworska, M. Verway, F. Tzelepis, A. Massoud, J. Gillard, G. Wong, G. Kobinger, Z. Xing, C. Couture, P. Joubert, J.H. Fritz, W.S. Powell, and M. Divangahi. 2014. Targeted prostaglandin E2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages. *Immunity* 40:554-568.
- Couper, K.N., D.G. Blount, and E.M. Riley. 2008. IL-10: the master regulator of immunity to infection. *Journal of immunology* 180:5771-5777.

- Cox, D.R., S.A. Smith, L.B. Epstein, and C.J. Epstein. 1984. Mouse trisomy 16 as an animal model of human trisomy 21 (Down syndrome): production of viable trisomy 16 diploid mouse chimeras. *Developmental biology* 101:416-424.
- Crotta, S., S. Davidson, T. Mahlakoiv, C.J. Desmet, M.R. Buckwalter, M.L. Albert, P. Staeheli, and A. Wack. 2013. Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS pathogens* 9:e1003773.
- Curtsinger, J.M., J.O. Valenzuela, P. Agarwal, D. Lins, and M.F. Mescher. 2005. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *Journal of immunology* 174:4465-4469.
- Dai, J., N.J. Megjugorac, S.B. Amrute, and P. Fitzgerald-Bocarsly. 2004. Regulation of IFN regulatory factor-7 and IFN-alpha production by enveloped virus and lipopolysaccharide in human plasmacytoid dendritic cells. *Journal of immunology* 173:1535-1548.
- Dai, J., N.J. Megjugorac, G.E. Gallagher, R.Y. Yu, and G. Gallagher. 2009. IFN-lambda1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. *Blood* 113:5829-5838.
- Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415-1421.
- Davidson, S., S. Crotta, T.M. McCabe, and A. Wack. 2014. Pathogenic potential of interferon alphabeta in acute influenza infection. *Nature communications* 5:3864.
- Davidson, S., G. Kaiko, Z. Loh, A. Lalwani, V. Zhang, K. Spann, S.Y. Foo, N. Hansbro, S. Uematsu, S. Akira, K.I. Matthaei, H.F. Rosenberg, P.S. Foster, and S. Phipps. 2011. Plasmacytoid dendritic cells promote host defense against acute pneumovirus infection via the TLR7-MyD88-dependent signaling pathway. *Journal of immunology* 186:5938-5948.
- Dawson, T.C., M.A. Beck, W.A. Kuziel, F. Henderson, and N. Maeda. 2000. Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. *The American journal of pathology* 156:1951-1959.
- de Groen, R.A., A. Boltjes, J. Hou, B.S. Liu, F. McPhee, J. Friberg, H.L. Janssen, and A. Boonstra. 2015. IFN-lambda-mediated IL-12 production in macrophages induces IFN-gamma production in human NK cells. *European journal of immunology* 45:250-259.
- de Hingh, Y.C., P.W. van der Vossen, E.F. Gemen, A.B. Mulder, W.C. Hop, F. Brus, and E. de Vries. 2005. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. *The Journal of pediatrics* 147:744-747.
- de Jong, M.D., C.P. Simmons, T.T. Thanh, V.M. Hien, G.J. Smith, T.N. Chau, D.M. Hoang, N.V. Chau, T.H. Khanh, V.C. Dong, P.T. Qui, B.V. Cam, Q. Ha do, Y. Guan, J.S. Peiris, N.T. Chinh, T.T. Hien, and J. Farrar. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nature medicine* 12:1203-1207.
- de Weerd, N.A., J.P. Vivian, T.K. Nguyen, N.E. Mangan, J.A. Gould, S.J. Braniff, L. Zaker-Tabrizi, K.Y. Fung, S.C. Forster, T. Beddoe, H.H. Reid, J. Rossjohn, and P.J. Hertzog. 2013. Structural basis of a unique interferon-beta signaling axis mediated via the receptor IFNAR1. *Nature immunology* 14:901-907.

- de Wit, E., Y. Kawaoka, M.D. de Jong, and R.A. Fouchier. 2008. Pathogenicity of highly pathogenic avian influenza virus in mammals. *Vaccine* 26 Suppl 4:D54-58.
- DeBerge, M.P., K.H. Ely, and R.I. Enelow. 2014. Soluble, but not transmembrane, TNF-alpha is required during influenza infection to limit the magnitude of immune responses and the extent of immunopathology. *Journal of immunology* 192:5839-5851.
- Dellgren, C., H.H. Gad, O.J. Hamming, J. Melchjorsen, and R. Hartmann. 2009. Human interferon-lambda3 is a potent member of the type III interferon family. *Genes and immunity* 10:125-131.
- Dergai, M., L. Tsyba, O. Dergai, I. Zlatskii, I. Skrypkin, V. Kovalenko, and A. Rynditch. 2010. Microexon-based regulation of ITSN1 and Src SH3 domains specificity relies on introduction of charged amino acids into the interaction interface. *Biochemical and biophysical research communications* 399:307-312.
- Desai, T.M., M. Marin, C.R. Chin, G. Savidis, A.L. Brass, and G.B. Melikyan. 2014. IFITM3 restricts influenza A virus entry by blocking the formation of fusion pores following virus-endosome hemifusion. *PLoS pathogens* 10:e1004048.
- Dessing, M.C., K.F. van der Sluijs, S. Florquin, and T. van der Poll. 2007. Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia. *Clinical immunology* 125:328-336.
- Diebold, S.S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303:1529-1531.
- Diebold, S.S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L.E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424:324-328.
- DiLillo, D.J., G.S. Tan, P. Palese, and J.V. Ravetch. 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. *Nature medicine* 20:143-151.
- Doherty, P.C., S.J. Turner, R.G. Webby, and P.G. Thomas. 2006. Influenza and the challenge for immunology. *Nature immunology* 7:449-455.
- Donelan, N.R., C.F. Basler, and A. Garcia-Sastre. 2003. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. *Journal of virology* 77:13257-13266.
- Dufour, J.H., M. Dziejman, M.T. Liu, J.H. Leung, T.E. Lane, and A.D. Luster. 2002. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *Journal of immunology* 168:3195-3204.
- Duncan, C.J., S.M. Mohamad, D.F. Young, A.J. Skelton, T.R. Leahy, D.C. Munday, K.M. Butler, S. Morfopoulou, J.R. Brown, M. Hubank, J. Connell, P.J. Gavin, C. McMahon, E. Dempsey, N.E. Lynch, T.S. Jacques, M. Valappil, A.J. Cant, J. Breuer, K.R. Engelhardt, R.E. Randall, and S. Hambleton. 2015. Human IFNAR2 deficiency: Lessons for antiviral immunity. *Science translational medicine* 7:307ra154.
- Dupuis, S., E. Jouanguy, S. Al-Hajjar, C. Fieschi, I.Z. Al-Mohsen, S. Al-Jumaah, K. Yang, A. Chapgier, C. Eidenschenk, P. Eid, A. Al Ghonaium, H. Tufenkeji, H. Frayha,

- S. Al-Gazlan, H. Al-Rayes, R.D. Schreiber, I. Gresser, and J.L. Casanova. 2003. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nature genetics* 33:388-391.
- Durbin, J.E., A. Fernandez-Sesma, C.K. Lee, T.D. Rao, A.B. Frey, T.M. Moran, S. Vukmanovic, A. Garcia-Sastre, and D.E. Levy. 2000. Type I IFN modulates innate and specific antiviral immunity. *Journal of immunology* 164:4220-4228.
- Edenborough, K.M., B.P. Gilbertson, and L.E. Brown. 2012. A mouse model for the study of contact-dependent transmission of influenza A virus and the factors that govern transmissibility. *Journal of virology* 86:12544-12551.
- Egli, A., D.M. Santer, D. O'Shea, K. Barakat, M. Syedbasha, M. Vollmer, A. Baluch, R. Bhat, J. Groenendyk, M.A. Joyce, L.F. Lisboa, B.S. Thomas, M. Battegay, N. Khanna, T. Mueller, D.L. Tyrrell, M. Houghton, A. Humar, and D. Kumar. 2014a. IL-28B is a key regulator of B- and T-cell vaccine responses against influenza. *PLoS pathogens* 10:e1004556.
- Egli, A., D.M. Santer, D. O'Shea, D.L. Tyrrell, and M. Houghton. 2014b. The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. *Emerging microbes & infections* 3:e51.
- Egorov, A., S. Brandt, S. Sereinig, J. Romanova, B. Ferko, D. Katinger, A. Grassauer, G. Alexandrova, H. Katinger, and T. Muster. 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. *Journal of virology* 72:6437-6441.
- Ehrhardt, C., R. Seyer, E.R. Hrinicius, T. Eierhoff, T. Wolff, and S. Ludwig. 2010. Interplay between influenza A virus and the innate immune signaling. *Microbes and infection / Institut Pasteur* 12:81-87.
- Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P.C. Doherty. 1991a. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *The Journal of experimental medicine* 174:875-880.
- Eichelberger, M.C., M.L. Wang, W. Allan, R.G. Webster, and P.C. Doherty. 1991b. Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. *The Journal of general virology* 72 (Pt 7):1695-1698.
- Ekiert, D.C., G. Bhabha, M.A. Elsliger, R.H. Friesen, M. Jongeneelen, M. Throsby, J. Goudsmit, and I.A. Wilson. 2009. Antibody recognition of a highly conserved influenza virus epitope. *Science* 324:246-251.
- Ekiert, D.C., R.H. Friesen, G. Bhabha, T. Kwaks, M. Jongeneelen, W. Yu, C. Ophorst, F. Cox, H.J. Korse, B. Brandenburg, R. Vogels, J.P. Brakenhoff, R. Kompier, M.H. Koldijk, L.A. Cornelissen, L.L. Poon, M. Peiris, W. Koudstaal, I.A. Wilson, and J. Goudsmit. 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 333:843-850.
- Ellis, G.T., S. Davidson, S. Crotta, N. Branzk, V. Papayannopoulos, and A. Wack. 2015. TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection. *EMBO reports* 16:1203-1218.
- Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicologic pathology* 35:495-516.
- Epstein, C.J., N.H. McManus, L.B. Epstein, A.A. Branca, S.B. D'Alessandro, and C. Baglioni. 1982. Direct evidence that the gene product of the human

- chromosome 21 locus, IFRC, is the interferon-alpha receptor. *Biochemical and biophysical research communications* 107:1060-1066.
- Epstein, L.B., and C.J. Epstein. 1976. Localization of the gene AVG for the antiviral expression of immune and classical interferon to the distal portion of the long arm of chromosome 21. *The Journal of infectious diseases* 133 Suppl:A56-62.
- Epstein, L.B., S.H. Lee, and C.J. Epstein. 1980. Enhanced sensitivity of trisomy 21 monocytes to the maturation-inhibiting effect of interferon. *Cellular immunology* 50:191-194.
- Epstein, S.L., C.Y. Lo, J.A. Misplon, and J.R. Bennink. 1998. Mechanism of protective immunity against influenza virus infection in mice without antibodies. *Journal of immunology* 160:322-327.
- Esposito, S., C.G. Molteni, S. Giliani, C. Mazza, A. Scala, L. Tagliaferri, C. Pelucchi, E. Fossali, A. Plebani, and N. Principi. 2012. Toll-like receptor 3 gene polymorphisms and severity of pandemic A/H1N1/2009 influenza in otherwise healthy children. *Virology journal* 9:270.
- Everitt, A.R., S. Clare, T. Pertel, S.P. John, R.S. Wash, S.E. Smith, C.R. Chin, E.M. Feeley, J.S. Sims, D.J. Adams, H.M. Wise, L. Kane, D. Goulding, P. Digard, V. Anttila, J.K. Baillie, T.S. Walsh, D.A. Hume, A. Palotie, Y. Xue, V. Colonna, C. Tyler-Smith, J. Dunning, S.B. Gordon, I.I. Gen, M. Investigators, R.L. Smyth, P.J. Openshaw, G. Dougan, A.L. Brass, and P. Kellam. 2012. IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484:519-523.
- Facchetti, F., C. De Wolf-Peeters, C. Kennes, G. Rossi, R. De Vos, J.J. van den Oord, and V.J. Desmet. 1990. Leukemia-associated lymph node infiltrates of plasmacytoid monocytes (so-called plasmacytoid T-cells). Evidence for two distinct histological and immunophenotypical patterns. *The American journal of surgical pathology* 14:101-112.
- Facchetti, F., C. de Wolf-Peeters, D.Y. Mason, K. Pulford, J.J. van den Oord, and V.J. Desmet. 1988. Plasmacytoid T cells. Immunohistochemical evidence for their monocyte/macrophage origin. *The American journal of pathology* 133:15-21.
- Feldman, M., and P. Fitzgerald-Bocarsly. 1990. Sequential enrichment and immunocytochemical visualization of human interferon-alpha-producing cells. *Journal of interferon research* 10:435-446.
- Ferdinands, J.M., A.M. Denison, N.F. Dowling, H.A. Jost, M.L. Gwinn, L. Liu, S.R. Zaki, and D.K. Shay. 2011. A pilot study of host genetic variants associated with influenza-associated deaths among children and young adults. *Emerging infectious diseases* 17:2294-2302.
- Fernandez Gonzalez, S., J.P. Jayasekera, and M.C. Carroll. 2008. Complement and natural antibody are required in the long-term memory response to influenza virus. *Vaccine* 26 Suppl 8:I86-93.
- Ferris, M.T., D.L. Aylor, D. Bottomly, A.C. Whitmore, L.D. Aicher, T.A. Bell, B. Bradet-Tretheway, J.T. Bryan, R.J. Buus, L.E. Gralinski, B.L. Haagmans, L. McMillan, D.R. Miller, E. Rosenzweig, W. Valdar, J. Wang, G.A. Churchill, D.W. Threadgill, S.K. McWeeney, M.G. Katze, F. Pardo-Manuel de Villena, R.S. Baric, and M.T. Heise. 2013. Modeling host genetic regulation of influenza pathogenesis in the collaborative cross. *PLoS pathogens* 9:e1003196.

- Fesq, H., M. Bacher, M. Nain, and D. Gems. 1994. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. *Immunobiology* 190:175-182.
- Finter, N.B., S. Chapman, P. Dowd, J.M. Johnston, V. Manna, N. Sarantis, N. Sheron, G. Scott, S. Phua, and P.B. Tatum. 1991. The use of interferon-alpha in virus infections. *Drugs* 42:749-765.
- Fleetwood, A.J., H. Dinh, A.D. Cook, P.J. Hertzog, and J.A. Hamilton. 2009. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *Journal of leukocyte biology* 86:411-421.
- Franchi, L., T. Eigenbrod, R. Munoz-Planillo, and G. Nunez. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature immunology* 10:241-247.
- Francis, T., and T.P. Magill. 1935. Immunological Studies with the Virus of Influenza. *The Journal of experimental medicine* 62:505-516.
- Friedli, M., M. Guipponi, S. Bertrand, D. Bertrand, M. Neerman-Arbez, H.S. Scott, S.E. Antonarakis, and A. Reymond. 2003. Identification of a novel member of the CLIC family, CLIC6, mapping to 21q22.12. *Gene* 320:31-40.
- Fujikura, D., S. Chiba, D. Muramatsu, M. Kazumata, Y. Nakayama, T. Kawai, S. Akira, H. Kida, and T. Miyazaki. 2013. Type-I interferon is critical for FasL expression on lung cells to determine the severity of influenza. *PloS one* 8:e55321.
- Fujimoto, I., T. Takizawa, Y. Ohba, and Y. Nakanishi. 1998. Co-expression of Fas and Fas-ligand on the surface of influenza virus-infected cells. *Cell death and differentiation* 5:426-431.
- Gack, M.U., Y.C. Shin, C.H. Joo, T. Urano, C. Liang, L. Sun, O. Takeuchi, S. Akira, Z. Chen, S. Inoue, and J.U. Jung. 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446:916-920.
- Garcia-Sastre, A., and C.A. Biron. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 312:879-882.
- Garcia-Sastre, A., R.K. Durbin, H. Zheng, P. Palese, R. Gertner, D.E. Levy, and J.E. Durbin. 1998a. The role of interferon in influenza virus tissue tropism. *Journal of virology* 72:8550-8558.
- Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D.E. Levy, J.E. Durbin, P. Palese, and T. Muster. 1998b. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252:324-330.
- Gardiner, K., D. Slavov, L. Bechtel, and M. Davisson. 2002. Annotation of human chromosome 21 for relevance to Down syndrome: gene structure and expression analysis. *Genomics* 79:833-843.
- Garrison, M.M., H. Jeffries, and D.A. Christakis. 2005. Risk of death for children with down syndrome and sepsis. *The Journal of pediatrics* 147:748-752.
- Geissmann, F., M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, and K. Ley. 2010. Development of monocytes, macrophages, and dendritic cells. *Science* 327:656-661.
- Gene, N. 2015. Flt3l FMS-like tyrosine kinase 3 ligand [Mus musculus (house mouse)] In <http://www.ncbi.nlm.nih.gov/gene/14256>.
- Gerhard, W. 2001. The role of the antibody response in influenza virus infection. *Current topics in microbiology and immunology* 260:171-190.

- GeurtsvanKessel, C.H., M.A. Willart, L.S. van Rijt, F. Muskens, M. Kool, C. Baas, K. Thielemans, C. Bennett, B.E. Clausen, H.C. Hoogsteden, A.D. Osterhaus, G.F. Rimmelzwaan, and B.N. Lambrecht. 2008. Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *The Journal of experimental medicine* 205:1621-1634.
- Gong, J.H., H. Sprenger, F. Hinder, A. Bender, A. Schmidt, S. Horch, M. Nain, and D. Gemsa. 1991. Influenza A virus infection of macrophages. Enhanced tumor necrosis factor-alpha (TNF-alpha) gene expression and lipopolysaccharide-triggered TNF-alpha release. *Journal of immunology* 147:3507-3513.
- Gorden, K.K., X.X. Qiu, C.C. Binsfeld, J.P. Vasilakos, and S.S. Alkan. 2006. Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. *Journal of immunology* 177:6584-6587.
- Goritzka, M., S. Makris, F. Kausar, L.R. Durant, C. Pereira, Y. Kumagai, F.J. Culley, M. Mack, S. Akira, and C. Johansson. 2015. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *The Journal of experimental medicine* 212:699-714.
- Gotch, F., A. McMichael, G. Smith, and B. Moss. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *The Journal of experimental medicine* 165:408-416.
- Gotoh, K., Y. Tanaka, A. Nishikimi, R. Nakamura, H. Yamada, N. Maeda, T. Ishikawa, K. Hoshino, T. Uruno, Q. Cao, S. Higashi, Y. Kawaguchi, M. Enjoji, R. Takayanagi, T. Kaisho, Y. Yoshikai, and Y. Fukui. 2010. Selective control of type I IFN induction by the Rac activator DOCK2 during TLR-mediated plasmacytoid dendritic cell activation. *The Journal of experimental medicine* 207:721-730.
- Gottfredsson, M., B.V. Halldorsson, S. Jonsson, M. Kristjansson, K. Kristjansson, K.G. Kristinsson, A. Love, T. Blondal, C. Viboud, S. Thorvaldsson, A. Helgason, J.R. Gulcher, K. Stefansson, and I. Jonsdottir. 2008. Lessons from the past: familial aggregation analysis of fatal pandemic influenza (Spanish flu) in Iceland in 1918. *Proceedings of the National Academy of Sciences of the United States of America* 105:1303-1308.
- Goubau, D., M. Schlee, S. Deddouche, A.J. Pruijssers, T. Zillinger, M. Goldeck, C. Schuberth, A.G. Van der Veen, T. Fujimura, J. Rehwinkel, J.A. Iskarpatyoti, W. Barchet, J. Ludwig, T.S. Dermody, G. Hartmann, and C. Reis e Sousa. 2014. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature* 514:372-375.
- Gough, D.J., N.L. Messina, L. Hii, J.A. Gould, K. Sabapathy, A.P. Robertson, J.A. Trapani, D.E. Levy, P.J. Hertzog, C.J. Clarke, and R.W. Johnstone. 2010. Functional crosstalk between type I and II interferon through the regulated expression of STAT1. *PLoS biology* 8:e1000361.
- Govorkova, E.A., N.A. Ilyushina, D.A. Boltz, A. Douglas, N. Yilmaz, and R.G. Webster. 2007. Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza virus. *Antimicrobial agents and chemotherapy* 51:1414-1424.
- Graham, M.B., D.K. Dalton, D. Giltinan, V.L. Braciale, T.A. Stewart, and T.J. Braciale. 1993. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. *The Journal of experimental medicine* 178:1725-1732.

- Grouard, G., M.C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *The Journal of experimental medicine* 185:1101-1111.
- Guo, H., J. Zhang, X. Zhang, Y. Wang, H. Yu, X. Yin, J. Li, P. Du, J. Plumas, L. Chaperot, J. Chen, L. Su, Y. Liu, and L. Zhang. 2015. SCARB2/LIMP-2 Regulates IFN Production of Plasmacytoid Dendritic Cells by Mediating Endosomal Translocation of TLR9 and Nuclear Translocation of IRF7. *Journal of immunology* 194:4737-4749.
- Hale, B.G., R.E. Randall, J. Ortin, and D. Jackson. 2008. The multifunctional NS1 protein of influenza A viruses. *The Journal of general virology* 89:2359-2376.
- Haller, O., H. Arnheiter, I. Gresser, and J. Lindenmann. 1979. Genetically determined, interferon-dependent resistance to influenza virus in mice. *The Journal of experimental medicine* 149:601-612.
- Hamada, H., E. Bassity, A. Flies, T.M. Strutt, L. Garcia-Hernandez Mde, K.K. McKinstry, T. Zou, S.L. Swain, and R.W. Dutton. 2013. Multiple redundant effector mechanisms of CD8+ T cells protect against influenza infection. *Journal of immunology* 190:296-306.
- Hamilton-Easton, A., and M. Eichelberger. 1995. Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice. *Journal of virology* 69:6359-6366.
- Hamming, O.J., E. Terczynska-Dyla, G. Vieyres, R. Dijkman, S.E. Jorgensen, H. Akhtar, P. Siupka, T. Pietschmann, V. Thiel, and R. Hartmann. 2013. Interferon lambda 4 signals via the IFNlambda receptor to regulate antiviral activity against HCV and coronaviruses. *The EMBO journal* 32:3055-3065.
- Hammond, M.E., G.R. Lapointe, P.H. Feucht, S. Hilt, C.A. Gallegos, C.A. Gordon, M.A. Giedlin, G. Mullenbach, and P. Tekamp-Olson. 1995. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *Journal of immunology* 155:1428-1433.
- Hancock, K., V. Veguilla, X. Lu, W. Zhong, E.N. Butler, H. Sun, F. Liu, L. Dong, J.R. DeVos, P.M. Gargiullo, T.L. Brammer, N.J. Cox, T.M. Tumpey, and J.M. Katz. 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *The New England journal of medicine* 361:1945-1952.
- Hardy, A.W., D.R. Graham, G.M. Shearer, and J.P. Herbeval. 2007. HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by Toll-like receptor 7-induced IFN-alpha. *Proceedings of the National Academy of Sciences of the United States of America* 104:17453-17458.
- Hashimoto, G., P.F. Wright, and D.T. Karzon. 1983. Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells. *The Journal of infectious diseases* 148:785-794.
- Hashimoto, Y., T. Moki, T. Takizawa, A. Shiratsuchi, and Y. Nakanishi. 2007. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. *Journal of immunology* 178:2448-2457.
- Havenar-Daughton, C., G.A. Kolumam, and K. Murali-Krishna. 2006. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal

- expansion in response to a viral but not a bacterial infection. *Journal of immunology* 176:3315-3319.
- Hayden, F.G., R. Fritz, M.C. Lobo, W. Alvord, W. Strober, and S.E. Straus. 1998. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *The Journal of clinical investigation* 101:643-649.
- Heer, A.K., A. Shamshiev, A. Donda, S. Uematsu, S. Akira, M. Kopf, and B.J. Marsland. 2007. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *Journal of immunology* 178:2182-2191.
- Herbeuval, J.P., J. Nilsson, A. Boasso, A.W. Hardy, M.J. Kruhlak, S.A. Anderson, M.J. Dolan, M. Dy, J. Andersson, and G.M. Shearer. 2006. Differential expression of IFN- α and TRAIL/DR5 in lymphoid tissue of progressor versus nonprogressor HIV-1-infected patients. *Proceedings of the National Academy of Sciences of the United States of America* 103:7000-7005.
- Herlocher, M.L., S. Elias, R. Truscon, S. Harrison, D. Mindell, C. Simon, and A.S. Monto. 2001. Ferrets as a transmission model for influenza: sequence changes in HA1 of type A (H3N2) virus. *The Journal of infectious diseases* 184:542-546.
- Herold, S., M. Steinmueller, W. von Wulffen, L. Cakarova, R. Pinto, S. Pleschka, M. Mack, W.A. Kuziel, N. Corazza, T. Brunner, W. Seeger, and J. Lohmeyer. 2008. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *The Journal of experimental medicine* 205:3065-3077.
- Hertz, T., C.M. Oshansky, P.L. Roddam, J.P. DeVincenzo, M.A. Caniza, N. Jojic, S. Mallal, E. Phillips, I. James, M.E. Halloran, P.G. Thomas, and L. Corey. 2013. HLA targeting efficiency correlates with human T-cell response magnitude and with mortality from influenza A infection. *Proceedings of the National Academy of Sciences of the United States of America* 110:13492-13497.
- Hill, D.A., G. Gridley, S. Cnattingius, L. Mellekjaer, M. Linet, H.O. Adami, J.H. Olsen, O. Nyren, and J.F. Fraumeni, Jr. 2003. Mortality and cancer incidence among individuals with Down syndrome. *Archives of internal medicine* 163:705-711.
- Hilton, J.M., D.A. Fitzgerald, and D.M. Cooper. 1999. Respiratory morbidity of hospitalized children with Trisomy 21. *Journal of paediatrics and child health* 35:383-386.
- Hofmann, P., H. Sprenger, A. Kaufmann, A. Bender, C. Hasse, M. Nain, and D. Gemsa. 1997. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. *Journal of leukocyte biology* 61:408-414.
- Hogner, K., T. Wolff, S. Pleschka, S. Plog, A.D. Gruber, U. Kalinke, H.D. Walmrath, J. Bodner, S. Gattenlohner, P. Lewe-Schlosser, M. Matrosovich, W. Seeger, J. Lohmeyer, and S. Herold. 2013. Macrophage-expressed IFN- β contributes to apoptotic alveolar epithelial cell injury in severe influenza virus pneumonia. *PLoS pathogens* 9:e1003188.
- Holoch, P.A., and T.S. Griffith. 2009. TNF-related apoptosis-inducing ligand (TRAIL): a new path to anti-cancer therapies. *European journal of pharmacology* 625:63-72.

- Holsinger, L.J., and R.A. Lamb. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology* 183:32-43.
- Holt, P.G., D.H. Strickland, M.E. Wikstrom, and F.L. Jahnsen. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nature reviews. Immunology* 8:142-152.
- Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, and T. Taniguchi. 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 434:1035-1040.
- Horby, P., N.Y. Nguyen, S.J. Dunstan, and J.K. Baillie. 2012. The role of host genetics in susceptibility to influenza: a systematic review. *PloS one* 7:e33180.
- Horby, P., N.Y. Nguyen, S.J. Dunstan, and J.K. Baillie. 2013. An updated systematic review of the role of host genetics in susceptibility to influenza. *Influenza and other respiratory viruses* 7 Suppl 2:37-41.
- Horby, P., H. Sudoyo, V. Viprakasit, A. Fox, P.Q. Thai, H. Yu, S. Davila, M. Hibberd, S.J. Dunstan, Y. Monteerarat, J.J. Farrar, S. Marzuki, and N.T. Hien. 2010. What is the evidence of a role for host genetics in susceptibility to influenza A/H5N1? *Epidemiology and infection* 138:1550-1558.
- Horisberger, M.A., P. Staeheli, and O. Haller. 1983. Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* 80:1910-1914.
- Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K.K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314:994-997.
- Huangfu, W.C., J. Qian, C. Liu, J. Liu, A.E. Lokshin, D.P. Baker, H. Rui, and S.Y. Fuchs. 2012. Inflammatory signaling compromises cell responses to interferon alpha. *Oncogene* 31:161-172.
- Hubbard, J.J., T. Greenwell-Wild, L. Barrett, J. Yang, R.A. Lempicki, S.M. Wahl, D.M. Asmuth, R.L. Murphy, R.B. Pollard, and S. Kottlil. 2012. Host gene expression changes correlating with anti-HIV-1 effects in human subjects after treatment with peginterferon Alfa-2a. *The Journal of infectious diseases* 205:1443-1447.
- Hummelshoj, L., L.P. Ryder, and L.K. Poulsen. 2006. The role of the interleukin-10 subfamily members in immunoglobulin production by human B cells. *Scandinavian journal of immunology* 64:40-47.
- Husser, L., M.P. Alves, N. Ruggli, and A. Summerfield. 2011. Identification of the role of RIG-I, MDA-5 and TLR3 in sensing RNA viruses in porcine epithelial cells using lentivirus-driven RNA interference. *Virus research* 159:9-16.
- Hwang, I., J.M. Scott, T. Kakarla, D.M. Duriancik, S. Choi, C. Cho, T. Lee, H. Park, A.R. French, E. Beli, E. Gardner, and S. Kim. 2012. Activation mechanisms of natural killer cells during influenza virus infection. *PloS one* 7:e51858.
- Ichinohe, T., I.K. Pang, and A. Iwasaki. 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nature immunology* 11:404-410.
- Ichinohe, T., I.K. Pang, Y. Kumamoto, D.R. Peaper, J.H. Ho, T.S. Murray, and A. Iwasaki. 2011. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 108:5354-5359.

- Imai, Y., K. Kuba, G.G. Neely, R. Yaghubian-Malhami, T. Perkmann, G. van Loo, M. Ermolaeva, R. Veldhuizen, Y.H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J.S. Peiris, A.S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C.J. Binder, and J.M. Penninger. 2008. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 133:235-249.
- Ioannidis, I., F. Ye, B. McNally, M. Willette, and E. Flano. 2013. Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. *Journal of virology* 87:3261-3270.
- Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proceedings of the Royal Society of London. Series B, Biological sciences* 147:258-267.
- Ishikawa, E., M. Nakazawa, M. Yoshinari, and M. Minami. 2005. Role of tumor necrosis factor-related apoptosis-inducing ligand in immune response to influenza virus infection in mice. *Journal of virology* 79:7658-7663.
- Ito, T., R. Amakawa, M. Inaba, S. Ikehara, K. Inaba, and S. Fukuhara. 2001. Differential regulation of human blood dendritic cell subsets by IFNs. *Journal of immunology* 166:2961-2969.
- Ivashkiv, L.B., and L.T. Donlin. 2014. Regulation of type I interferon responses. *Nature reviews. Immunology* 14:36-49.
- Izaguirre, A., B.J. Barnes, S. Amrute, W.S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P.M. Pitha, and P. Fitzgerald-Bocarsly. 2003. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *Journal of leukocyte biology* 74:1125-1138.
- Jaitin, D.A., L.C. Roisman, E. Jaks, M. Gavutis, J. Piehler, J. Van der Heyden, G. Uze, and G. Schreiber. 2006. Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. *Molecular and cellular biology* 26:1888-1897.
- Jameson, J., J. Cruz, and F.A. Ennis. 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *Journal of virology* 72:8682-8689.
- Janeway, C.A., Travers, Paul, Walport Mark. 1999. *Immuno Biology*. 4:
- Jayaraman, A., A. Chandrasekaran, K. Viswanathan, R. Raman, J.G. Fox, and R. Sasisekharan. 2012. Decoding the distribution of glycan receptors for human-adapted influenza A viruses in ferret respiratory tract. *PloS one* 7:e27517.
- Jayasekera, J.P., E.A. Moseman, and M.C. Carroll. 2007. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of virology* 81:3487-3494.
- Jayasekera, J.P., C.G. Vinuesa, G. Karupiah, and N.J. King. 2006. Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice. *The Journal of general virology* 87:3361-3371.
- Jefferson, T.O., V. Demicheli, C. Di Pietrantonj, M. Jones, and D. Rivetti. 2006. Neuraminidase inhibitors for preventing and treating influenza in healthy adults. *The Cochrane database of systematic reviews* CD001265.
- Jeisy-Scott, V., J.H. Kim, W.G. Davis, W. Cao, J.M. Katz, and S. Sambhara. 2012. TLR7 recognition is dispensable for influenza virus A infection but important for

- the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. *Journal of virology* 86:10988-10998.
- Jewell, N.A., N. Vaghefi, S.E. Mertz, P. Akter, R.S. Peebles, Jr., L.O. Bakaletz, R.K. Durbin, E. Flano, and J.E. Durbin. 2007. Differential type I interferon induction by respiratory syncytial virus and influenza A virus in vivo. *Journal of virology* 81:9790-9800.
- Jiang, L.J., N.N. Zhang, F. Ding, X.Y. Li, L. Chen, H.X. Zhang, W. Zhang, S.J. Chen, Z.G. Wang, J.M. Li, Z. Chen, and J. Zhu. 2011. RA-inducible gene-I induction augments STAT1 activation to inhibit leukemia cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 108:1897-1902.
- Jilg, N., W. Lin, J. Hong, E.A. Schaefer, D. Wolski, J. Meixong, K. Goto, C. Brisac, P. Chusri, D.N. Fusco, S. Chevaliez, J. Luther, K. Kumthip, T.J. Urban, L.F. Peng, G.M. Lauer, and R.T. Chung. 2014. Kinetic differences in the induction of interferon stimulated genes by interferon-alpha and interleukin 28B are altered by infection with hepatitis C virus. *Hepatology* 59:1250-1261.
- Jordan, W.J., J. Eskdale, M. Boniotto, M. Rodia, D. Kellner, and G. Gallagher. 2007. Modulation of the human cytokine response by interferon lambda-1 (IFN-lambda1/IL-29). *Genes and immunity* 8:13-20.
- Julkunen, I., K. Melen, M. Nyqvist, J. Pirhonen, T. Sareneva, and S. Matikainen. 2000. Inflammatory responses in influenza A virus infection. *Vaccine* 19 Suppl 1:S32-37.
- Julkunen, I., T. Sareneva, J. Pirhonen, T. Ronni, K. Melen, and S. Matikainen. 2001. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine & growth factor reviews* 12:171-180.
- Kadowaki, N., S. Antonenko, J.Y. Lau, and Y.J. Liu. 2000. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *The Journal of experimental medicine* 192:219-226.
- Kaiser, L., R.S. Fritz, S.E. Straus, L. Gubareva, and F.G. Hayden. 2001. Symptom pathogenesis during acute influenza: interleukin-6 and other cytokine responses. *Journal of medical virology* 64:262-268.
- Kalie, E., D.A. Jaitin, Y. Podoplelova, J. Piehler, and G. Schreiber. 2008. The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. *The Journal of biological chemistry* 283:32925-32936.
- Kallfass, C., S. Lienenklaus, S. Weiss, and P. Staeheli. 2013. Visualizing the beta interferon response in mice during infection with influenza A viruses expressing or lacking nonstructural protein 1. *Journal of virology* 87:6925-6930.
- Kaminski, M.M., A. Ohnemus, M. Cornitescu, and P. Staeheli. 2012. Plasmacytoid dendritic cells and Toll-like receptor 7-dependent signalling promote efficient protection of mice against highly virulent influenza A virus. *The Journal of general virology* 93:555-559.
- Kash, J.C., T.M. Tumpey, S.C. Proll, V. Carter, O. Perwitasari, M.J. Thomas, C.F. Basler, P. Palese, J.K. Taubenberger, A. Garcia-Sastre, D.E. Swayne, and M.G. Katze. 2006. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443:578-581.

- Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23:19-28.
- Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K.J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C.S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101-105.
- Kaufmann, A., R. Salentin, R.G. Meyer, D. Bussfeld, C. Pauligk, H. Fesq, P. Hofmann, M. Nain, D. Gemsa, and H. Sprenger. 2001. Defense against influenza A virus infection: essential role of the chemokine system. *Immunobiology* 204:603-613.
- Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637-650.
- Kennedy, D.W., and J.L. Abkowitz. 1997. Kinetics of central nervous system microglial and macrophage engraftment: analysis using a transgenic bone marrow transplantation model. *Blood* 90:986-993.
- Kerkmann, M., S. Rothenfusser, V. Hornung, A. Towarowski, M. Wagner, A. Sarris, T. Giese, S. Endres, and G. Hartmann. 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *Journal of immunology* 170:4465-4474.
- Key, F.M., B. Peter, M.Y. Dennis, E. Huerta-Sanchez, W. Tang, L. Prokunina-Olsson, R. Nielsen, and A.M. Andres. 2014. Selection on a variant associated with improved viral clearance drives local, adaptive pseudogenization of interferon lambda 4 (IFNL4). *PLoS genetics* 10:e1004681.
- Keynan, Y., J. Juno, A. Meyers, T.B. Ball, A. Kumar, E. Rubinstein, and K.R. Fowke. 2010. Chemokine receptor 5 big up tri, open32 allele in patients with severe pandemic (H1N1) 2009. *Emerging infectious diseases* 16:1621-1622.
- Khan, I.M., R.A. Fisher, K.J. Johnson, M.E. Bailey, M.J. Siciliano, A.M. Kessler, M. Farrer, B. Carritt, T. Kamalati, and L. Buluwela. 1994. The SON gene encodes a conserved DNA binding protein mapping to human chromosome 21. *Annals of human genetics* 58:25-34.
- Kim, H.M., Y.W. Lee, K.J. Lee, H.S. Kim, S.W. Cho, N. van Rooijen, Y. Guan, and S.H. Seo. 2008. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. *Journal of virology* 82:4265-4274.
- Kobasa, D., S.M. Jones, K. Shinya, J.C. Kash, J. Copps, H. Ebihara, Y. Hatta, J.H. Kim, P. Halfmann, M. Hatta, F. Feldmann, J.B. Alimonti, L. Fernando, Y. Li, M.G. Katze, H. Feldmann, and Y. Kawaoka. 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445:319-323.
- Kobasa, D., A. Takada, K. Shinya, M. Hatta, P. Halfmann, S. Theriault, H. Suzuki, H. Nishimura, K. Mitamura, N. Sugaya, T. Usui, T. Murata, Y. Maeda, S. Watanabe, M. Suresh, T. Suzuki, Y. Suzuki, H. Feldmann, and Y. Kawaoka. 2004. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature* 431:703-707.
- Kochs, G., A. Garcia-Sastre, and L. Martinez-Sobrido. 2007. Multiple anti-interferon actions of the influenza A virus NS1 protein. *Journal of virology* 81:7011-7021.

- Koerner, I., G. Kochs, U. Kalinke, S. Weiss, and P. Staeheli. 2007. Protective role of beta interferon in host defense against influenza A virus. *Journal of virology* 81:2025-2030.
- Koltsida, O., M. Hausding, A. Stavropoulos, S. Koch, G. Tzelepis, C. Ubel, S.V. Kotenko, P. Sideras, H.A. Lehr, M. Tepe, K.M. Klucher, S.E. Doyle, M.F. Neurath, S. Finotto, and E. Andreakos. 2011. IL-28A (IFN-lambda2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease. *EMBO molecular medicine* 3:348-361.
- Kotenko, S.V., G. Gallagher, V.V. Baurin, A. Lewis-Antes, M. Shen, N.K. Shah, J.A. Langer, F. Sheikh, H. Dickensheets, and R.P. Donnelly. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature immunology* 4:69-77.
- Koutsonanos, D.G., M. del Pilar Martin, V.G. Zarnitsyn, J. Jacob, M.R. Prausnitz, R.W. Compans, and I. Skountzou. 2011. Serological memory and long-term protection to novel H1N1 influenza virus after skin vaccination. *The Journal of infectious diseases* 204:582-591.
- Kowalinski, E., T. Lunardi, A.A. McCarthy, J. Louber, J. Brunel, B. Grigorov, D. Gerlier, and S. Cusack. 2011. Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell* 147:423-435.
- Koyama, S., K.J. Ishii, H. Kumar, T. Tanimoto, C. Coban, S. Uematsu, T. Kawai, and S. Akira. 2007. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. *Journal of immunology* 179:4711-4720.
- Kozak, W., V. Poli, D. Soszynski, C.A. Conn, L.R. Leon, and M.J. Kluger. 1997. Sickness behavior in mice deficient in interleukin-6 during turpentine abscess and influenza pneumonitis. *The American journal of physiology* 272:R621-630.
- Krammer, F., P. Palese, and J. Steel. 2015. Advances in universal influenza virus vaccine design and antibody mediated therapies based on conserved regions of the hemagglutinin. *Current topics in microbiology and immunology* 386:301-321.
- Kreijtz, J.H., R.A. Fouchier, and G.F. Rimmelzwaan. 2011. Immune responses to influenza virus infection. *Virus research* 162:19-30.
- Kugel, D., G. Kochs, K. Obojes, J. Roth, G.P. Kobinger, D. Kobasa, O. Haller, P. Staeheli, and V. von Messling. 2009. Intranasal administration of alpha interferon reduces seasonal influenza A virus morbidity in ferrets. *Journal of virology* 83:3843-3851.
- Kumagai, Y., H. Kumar, S. Koyama, T. Kawai, O. Takeuchi, and S. Akira. 2009. Cutting Edge: TLR-Dependent viral recognition along with type I IFN positive feedback signaling masks the requirement of viral replication for IFN- α production in plasmacytoid dendritic cells. *Journal of immunology* 182:3960-3964.
- Kumar, K.G., H. Barriere, C.J. Carbone, J. Liu, G. Swaminathan, P. Xu, Y. Li, D.P. Baker, J. Peng, G.L. Lukacs, and S.Y. Fuchs. 2007. Site-specific ubiquitination exposes a linear motif to promote interferon-alpha receptor endocytosis. *The Journal of cell biology* 179:935-950.
- Kumar, K.G., W. Tang, A.K. Ravindranath, W.A. Clark, E. Croze, and S.Y. Fuchs. 2003. SCF(HOS) ubiquitin ligase mediates the ligand-induced down-regulation of the interferon-alpha receptor. *The EMBO journal* 22:5480-5490.

- Kurihara, T., G. Warr, J. Loy, and R. Bravo. 1997. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *The Journal of experimental medicine* 186:1757-1762.
- Kusters, M.A., V.L. Bok, W.E. Bolz, E.G. Huijskens, M.F. Peeters, and E. de Vries. 2012. Influenza A/H1N1 vaccination response is inadequate in down syndrome children when the latest cut-off values are used. *The Pediatric infectious disease journal* 31:1284-1285.
- Kusters, M.A., E.F. Gemen, R.H. Verstegen, P.C. Wever, and D.E.V. E. 2010. Both normal memory counts and decreased naive cells favor intrinsic defect over early senescence of Down syndrome T lymphocytes. *Pediatric research* 67:557-562.
- Kuziel, W.A., S.J. Morgan, T.C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proceedings of the National Academy of Sciences of the United States of America* 94:12053-12058.
- Lamb, J.R., J.N. Woody, R.J. Hartzman, and D.D. Eckels. 1982. In vitro influenza virus-specific antibody production in man: antigen-specific and HLA-restricted induction of helper activity mediated by cloned human T lymphocytes. *Journal of immunology* 129:1465-1470.
- LaMere, M.W., H.T. Lam, A. Moquin, L. Haynes, F.E. Lund, T.D. Randall, and D.A. Kaminski. 2011. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *Journal of immunology* 186:4331-4339.
- Lane, H.C., V. Davey, J.A. Kovacs, J. Feinberg, J.A. Metcalf, B. Herpin, R. Walker, L. Deyton, R.T. Davey, Jr., J. Falloon, and et al. 1990. Interferon-alpha in patients with asymptomatic human immunodeficiency virus (HIV) infection. A randomized, placebo-controlled trial. *Annals of internal medicine* 112:805-811.
- Langlet, C., S. Tamoutounour, S. Henri, H. Luche, L. Ardouin, C. Gregoire, B. Malissen, and M. Guillemins. 2012. CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular immunization. *Journal of immunology* 188:1751-1760.
- Langlois, R.A., and K.L. Legge. 2010. Plasmacytoid dendritic cells enhance mortality during lethal influenza infections by eliminating virus-specific CD8 T cells. *Journal of immunology* 184:4440-4446.
- Lapenta, C., S.M. Santini, M. Logozzi, M. Spada, M. Andreotti, T. Di Pucchio, S. Parlato, and F. Belardelli. 2003. Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-alpha. *The Journal of experimental medicine* 198:361-367.
- Lauterbach, H., B. Bathke, S. Gilles, C. Traidl-Hoffmann, C.A. Luber, G. Fejer, M.A. Freudenberg, G.M. Davey, D. Vremec, A. Kallies, L. Wu, K. Shortman, P. Chaplin, M. Suter, M. O'Keeffe, and H. Hochrein. 2010. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *The Journal of experimental medicine* 207:2703-2717.
- Lavoie, T.B., E. Kalie, S. Crisafulli-Cabatu, R. Abramovich, G. DiGioia, K. Moolchan, S. Pestka, and G. Schreiber. 2011. Binding and activity of all human alpha interferon subtypes. *Cytokine* 56:282-289.
- Le Bon, A., C. Thompson, E. Kamphuis, V. Durand, C. Rossmann, U. Kalinke, and D.F. Tough. 2006. Cutting edge: enhancement of antibody responses through

- direct stimulation of B and T cells by type I IFN. *Journal of immunology* 176:2074-2078.
- Le Goffic, R., J. Pothlichet, D. Vitour, T. Fujita, E. Meurs, M. Chignard, and M. Si-Tahar. 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *Journal of immunology* 178:3368-3372.
- Lee, H.K., J.M. Lund, B. Ramanathan, N. Mizushima, and A. Iwasaki. 2007. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* 315:1398-1401.
- Lee, L.Y., L.A. Ha do, C. Simmons, M.D. de Jong, N.V. Chau, R. Schumacher, Y.C. Peng, A.J. McMichael, J.J. Farrar, G.L. Smith, A.R. Townsend, B.A. Askonas, S. Rowland-Jones, and T. Dong. 2008. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *The Journal of clinical investigation* 118:3478-3490.
- Lee, S.M., K.H. Kok, M. Jaume, T.K. Cheung, T.F. Yip, J.C. Lai, Y. Guan, R.G. Webster, D.Y. Jin, and J.S. Peiris. 2014. Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 111:3793-3798.
- Leung, H.S., O.T. Li, R.W. Chan, M.C. Chan, J.M. Nicholls, and L.L. Poon. 2012. Entry of influenza A Virus with a alpha2,6-linked sialic acid binding preference requires host fibronectin. *Journal of virology* 86:10704-10713.
- Li, C.R., J. Yang, S.L. Jia, G.B. Wang, Y.X. He, Y. Zu, W.G. Yang, and D. Fu. 2010. [Changes of immune function in patients with 2009 influenza A (H1N1)]. *Zhonghua er ke za zhi. Chinese journal of pediatrics* 48:947-953.
- Lichtner, M., C.M. Mastroianni, R. Rossi, G. Russo, V. Belvisi, R. Marocco, C. Mascia, C. Del Borgo, F. Mengoni, I. Sauzullo, G. d'Ettorre, C. D'Agostino, A.P. Massetti, and V. Vullo. 2011. Severe and persistent depletion of circulating plasmacytoid dendritic cells in patients with 2009 pandemic H1N1 infection. *PloS one* 6:e19872.
- Lin, K.L., Y. Suzuki, H. Nakano, E. Ramsburg, and M.D. Gunn. 2008. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. *Journal of immunology* 180:2562-2572.
- Lin, K.L., S. Sweeney, B.D. Kang, E. Ramsburg, and M.D. Gunn. 2011. CCR2-antagonist prophylaxis reduces pulmonary immune pathology and markedly improves survival during influenza infection. *Journal of immunology* 186:508-515.
- Lindenmann, J. 1962. Resistance of mice to mouse-adapted influenza A virus. *Virology* 16:203-204.
- Lindenmann, J., C.A. Lane, and D. Hobson. 1963. The Resistance of A2g Mice to Myxoviruses. *Journal of immunology* 90:942-951.
- Ling, P.D., M.K. Warren, and S.N. Vogel. 1985. Antagonistic effect of interferon-beta on the interferon-gamma-induced expression of Ia antigen in murine macrophages. *Journal of immunology* 135:1857-1863.
- Liu, B.S., H.L. Janssen, and A. Boonstra. 2011. IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. *Blood* 117:2385-2395.

- Liu, Y., S. Li, G. Zhang, G. Nie, Z. Meng, D. Mao, C. Chen, X. Chen, B. Zhou, and G. Zeng. 2013. Genetic variants in IL1A and IL1B contribute to the susceptibility to 2009 pandemic H1N1 influenza A virus. *BMC immunology* 14:37.
- Liu, Y.J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annual review of immunology* 23:275-306.
- Loo, Y.M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M.A. Gill, A. Garcia-Sastre, M.G. Katze, and M. Gale, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *Journal of virology* 82:335-345.
- Loo, Y.M., and M. Gale, Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34:680-692.
- Lopez, C.B., J.S. Yount, T. Hermesh, and T.M. Moran. 2006. Sendai virus infection induces efficient adaptive immunity independently of type I interferons. *Journal of virology* 80:4538-4545.
- Louie, J.K., M. Acosta, K. Winter, C. Jean, S. Gavali, R. Schechter, D. Vugia, K. Harriman, B. Matyas, C.A. Glaser, M.C. Samuel, J. Rosenberg, J. Talarico, D. Hatch, and G. California Pandemic Working. 2009. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. *Jama* 302:1896-1902.
- Ludwig, S., X. Wang, C. Ehrhardt, H. Zheng, N. Donelan, O. Planz, S. Pleschka, A. Garcia-Sastre, G. Heins, and T. Wolff. 2002. The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. *Journal of virology* 76:11166-11171.
- Lui, G., O. Manches, J. Angel, J.P. Molens, L. Chaperot, and J. Plumas. 2009. Plasmacytoid dendritic cells capture and cross-present viral antigens from influenza-virus exposed cells. *PloS one* 4:e7111.
- Lund, J.M., L. Alexopoulou, A. Sato, M. Karow, N.C. Adams, N.W. Gale, A. Iwasaki, and R.A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America* 101:5598-5603.
- Luo, M., X. Qu, R. Pan, D. Zhu, Y. Zhang, J. Wu, and Z. Pan. 2011. The virus-induced signaling adaptor molecule enhances DNA-raised immune protection against H5N1 influenza virus infection in mice. *Vaccine* 29:2561-2567.
- Ma, W., K.M. Lager, A.L. Vincent, B.H. Janke, M.R. Gramer, and J.A. Richt. 2009. The role of swine in the generation of novel influenza viruses. *Zoonoses and public health* 56:326-337.
- Maher, J.A., and J. DeStefano. 2004. The ferret: an animal model to study influenza virus. *Lab animal* 33:50-53.
- Mahieu, T., J.M. Park, H. Revets, B. Pasche, A. Lengeling, J. Staelens, A. Wullaert, I. Vanlaere, T. Hocheplied, F. van Roy, M. Karin, and C. Libert. 2006. The wild-derived inbred mouse strain SPRET/Ei is resistant to LPS and defective in IFN-beta production. *Proceedings of the National Academy of Sciences of the United States of America* 103:2292-2297.
- Mahlakoiv, T., P. Hernandez, K. Gronke, A. Diefenbach, and P. Staeheli. 2015. Leukocyte-derived IFN-alpha/beta and epithelial IFN-lambda constitute a compartmentalized mucosal defense system that restricts enteric virus infections. *PLoS pathogens* 11:e1004782.
- Maines, T.R., L.M. Chen, Y. Matsuoka, H. Chen, T. Rowe, J. Ortin, A. Falcon, T.H. Nguyen, Q. Mai le, E.R. Sedyaningsih, S. Harun, T.M. Tumpey, R.O. Donis, N.J.

- Cox, K. Subbarao, and J.M. Katz. 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. *Proceedings of the National Academy of Sciences of the United States of America* 103:12121-12126.
- Marie, I., J.E. Durbin, and D.E. Levy. 1998. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *The EMBO journal* 17:6660-6669.
- Martinez, J., X. Huang, and Y. Yang. 2010. Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* 107:6442-6447.
- Matikainen, S., J. Siren, J. Tissari, V. Veckman, J. Pirhonen, M. Severa, Q. Sun, R. Lin, S. Meri, G. Uze, J. Hiscott, and I. Julkunen. 2006. Tumor necrosis factor alpha enhances influenza A virus-induced expression of antiviral cytokines by activating RIG-I gene expression. *Journal of virology* 80:3515-3522.
- Matute-Bello, G., J.S. Lee, W.C. Liles, C.W. Frevert, S. Mongovin, V. Wong, K. Ballman, S. Sutlief, and T.R. Martin. 2005. Fas-mediated acute lung injury requires fas expression on nonmyeloid cells of the lung. *Journal of immunology* 175:4069-4075.
- Matute-Bello, G., W.C. Liles, C.W. Frevert, M. Nakamura, K. Ballman, C. Vathanaprida, P.A. Kiener, and T.R. Martin. 2001a. Recombinant human Fas ligand induces alveolar epithelial cell apoptosis and lung injury in rabbits. *American journal of physiology. Lung cellular and molecular physiology* 281:L328-335.
- Matute-Bello, G., R.K. Winn, M. Jonas, E.Y. Chi, T.R. Martin, and W.C. Liles. 2001b. Fas (CD95) induces alveolar epithelial cell apoptosis in vivo: implications for acute pulmonary inflammation. *The American journal of pathology* 158:153-161.
- Matzinger, S.R., T.D. Carroll, J.C. Dutra, Z.M. Ma, and C.J. Miller. 2013. Myxovirus resistance gene A (MxA) expression suppresses influenza A virus replication in alpha interferon-treated primate cells. *Journal of virology* 87:1150-1158.
- Mauad, T., L.A. Hajjar, G.D. Callegari, L.F. da Silva, D. Schout, F.R. Galas, V.A. Alves, D.M. Malheiros, J.O. Auler, Jr., A.F. Ferreira, M.R. Borsato, S.M. Bezerra, P.S. Gutierrez, E.T. Caldini, C.A. Pasqualucci, M. Dolhnikoff, and P.H. Saldiva. 2010. Lung pathology in fatal novel human influenza A (H1N1) infection. *American journal of respiratory and critical care medicine* 181:72-79.
- Mazanec, M.B., C.L. Coudret, and D.R. Fletcher. 1995. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. *Journal of virology* 69:1339-1343.
- McAuley, J.L., M.D. Tate, C.J. MacKenzie-Kludas, A. Pinar, W. Zeng, A. Stutz, E. Latz, L.E. Brown, and A. Mansell. 2013. Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. *PLoS pathogens* 9:e1003392.
- McKinlay, M.A. 2001. Recent advances in the treatment of rhinovirus infections. *Current opinion in pharmacology* 1:477-481.
- McKinstry, K.K., T.M. Strutt, A. Buck, J.D. Curtis, J.P. Dibble, G. Huston, M. Tighe, H. Hamada, S. Sell, R.W. Dutton, and S.L. Swain. 2009. IL-10 deficiency

- unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. *Journal of immunology* 182:7353-7363.
- McMichael, A. 1978. HLA restriction of human cytotoxic T lymphocytes specific for influenza virus. Poor recognition of virus associated with HLA A2. *The Journal of experimental medicine* 148:1458-1467.
- McMichael, A.J., F.M. Gotch, G.R. Noble, and P.A. Beare. 1983. Cytotoxic T-cell immunity to influenza. *The New England journal of medicine* 309:13-17.
- McMichael, A.J., A. Ting, H.J. Zweerink, and B.A. Askonas. 1977. HLA restriction of cell-mediated lysis of influenza virus-infected human cells. *Nature* 270:524-526.
- McNab, F.W., J. Ewbank, A. Howes, L. Moreira-Teixeira, A. Martirosyan, N. Ghilardi, M. Saraiva, and A. O'Garra. 2014. Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-gamma for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. *Journal of immunology* 193:3600-3612.
- Mendel, D.B., C.Y. Tai, P.A. Escarpe, W. Li, R.W. Sidwell, J.H. Huffman, C. Sweet, K.J. Jakeman, J. Merson, S.A. Lacy, W. Lew, M.A. Williams, L. Zhang, M.S. Chen, N. Bischofberger, and C.U. Kim. 1998. Oral administration of a prodrug of the influenza virus neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. *Antimicrobial agents and chemotherapy* 42:640-646.
- Minegishi, Y., M. Saito, T. Morio, K. Watanabe, K. Agematsu, S. Tsuchiya, H. Takada, T. Hara, N. Kawamura, T. Ariga, H. Kaneko, N. Kondo, I. Tsuge, A. Yachie, Y. Sakiyama, T. Iwata, F. Bessho, T. Ohishi, K. Joh, K. Imai, K. Kogawa, M. Shinohara, M. Fujieda, H. Wakiguchi, S. Pasic, M. Abinun, H.D. Ochs, E.D. Renner, A. Jansson, B.H. Belohradsky, A. Metin, N. Shimizu, S. Mizutani, T. Miyawaki, S. Nonoyama, and H. Karasuyama. 2006. Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. *Immunity* 25:745-755.
- Misharin, A.V., L. Morales-Nebreda, G.M. Mutlu, G.R. Budinger, and H. Perlman. 2013. Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung. *American journal of respiratory cell and molecular biology* 49:503-510.
- Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.
- Monsalvo, A.C., J.P. Batalle, M.F. Lopez, J.C. Krause, J. Klemenc, J.Z. Hernandez, B. Maskin, J. Bugna, C. Rubinstein, L. Aguilar, L. Dalurzo, R. Libster, V. Savy, E. Baumeister, L. Aguilar, G. Cabral, J. Font, L. Solari, K.P. Weller, J. Johnson, M. Echavarria, K.M. Edwards, J.D. Chappell, J.E. Crowe, Jr., J.V. Williams, G.A. Melendi, and F.P. Polack. 2011. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nature medicine* 17:195-199.
- Monticelli, L.A., G.F. Sonnenberg, M.C. Abt, T. Alenghat, C.G. Ziegler, T.A. Doering, J.M. Angelosanto, B.J. Laidlaw, C.Y. Yang, T. Sathaliyawala, M. Kubota, D. Turner, J.M. Diamond, A.W. Goldrath, D.L. Farber, R.G. Collman, E.J. Wherry, and D. Artis. 2011. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology* 12:1045-1054.

- Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D.F. Tough. 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99:3263-3271.
- Mordstein, M., G. Kochs, L. Dumoutier, J.C. Renauld, S.R. Paludan, K. Klucher, and P. Staeheli. 2008. Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. *PLoS pathogens* 4:e1000151.
- Mordstein, M., E. Neugebauer, V. Ditt, B. Jessen, T. Rieger, V. Falcone, F. Sorgeloos, S. Ehl, D. Mayer, G. Kochs, M. Schwemmle, S. Gunther, C. Drosten, T. Michiels, and P. Staeheli. 2010. Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *Journal of virology* 84:5670-5677.
- Morens, D.M., J.K. Taubenberger, and A.S. Fauci. 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *The Journal of infectious diseases* 198:962-970.
- Mozdzanowska, K., K. Maiese, M. Furchner, and W. Gerhard. 1999. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. *Virology* 254:138-146.
- Muir, A.J., S. Arora, G. Everson, R. Flisiak, J. George, R. Ghalib, S.C. Gordon, T. Gray, S. Greenbloom, T. Hassanein, J. Hillson, M.A. Horga, I.M. Jacobson, L. Jeffers, K.V. Kowdley, E. Lawitz, S. Lueth, M. Rodriguez-Torres, V. Rustgi, L. Shemanski, M.L. Shiffman, S. Srinivasan, H.E. Vargas, J.M. Vierling, D. Xu, J.C. Lopez-Talavera, S. Zeuzem, and E.s. group. 2014. A randomized phase 2b study of peginterferon lambda-1a for the treatment of chronic HCV infection. *Journal of hepatology* 61:1238-1246.
- Muller, U., U. Steinhoff, L.F. Reis, S. Hemmi, J. Pavlovic, R.M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918-1921.
- Murray, P.J. 2007. The JAK-STAT signaling pathway: input and output integration. *Journal of immunology* 178:2623-2629.
- Nagano, Y., Y. Kojima, and Y. Sawai. 1954. [Immunity and interference in vaccinia; inhibition of skin infection by inactivated virus]. *Comptes rendus des seances de la Societe de biologie et de ses filiales* 148:750-752.
- Nakano, H., M. Yanagita, and M.D. Gunn. 2001. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *The Journal of experimental medicine* 194:1171-1178.
- Napoli, J., G.A. Bishop, P.H. McGuinness, D.M. Painter, and G.W. McCaughan. 1996. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. *Hepatology* 24:759-765.
- Narasaraju, T., H.H. Ng, M.C. Phoon, and V.T. Chow. 2010. MCP-1 antibody treatment enhances damage and impedes repair of the alveolar epithelium in influenza pneumonitis. *American journal of respiratory cell and molecular biology* 42:732-743.
- Narasaraju, T., E. Yang, R.P. Samy, H.H. Ng, W.P. Poh, A.A. Liew, M.C. Phoon, N. van Rooijen, and V.T. Chow. 2011. Excessive neutrophils and neutrophil

- extracellular traps contribute to acute lung injury of influenza pneumonitis. *The American journal of pathology* 179:199-210.
- North, T.E., T. Stacy, C.J. Matheny, N.A. Speck, and M.F. de Bruijn. 2004. Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells. *Stem cells* 22:158-168.
- Novikov, A., M. Cardone, R. Thompson, K. Shenderov, K.D. Kirschman, K.D. Mayer-Barber, T.G. Myers, R.L. Rabin, G. Trinchieri, A. Sher, and C.G. Feng. 2011. Mycobacterium tuberculosis triggers host type I IFN signaling to regulate IL-1 β production in human macrophages. *Journal of immunology* 187:2540-2547.
- O'Brien, M., O. Manches, and N. Bhardwaj. 2013. Plasmacytoid dendritic cells in HIV infection. *Advances in experimental medicine and biology* 762:71-107.
- Okamoto, M., S. Kato, K. Oizumi, M. Kinoshita, Y. Inoue, K. Hoshino, S. Akira, A.N. McKenzie, H.A. Young, and T. Hoshino. 2002. Interleukin 18 (IL-18) in synergy with IL-2 induces lethal lung injury in mice: a potential role for cytokines, chemokines, and natural killer cells in the pathogenesis of interstitial pneumonia. *Blood* 99:1289-1298.
- Olsen, B., V.J. Munster, A. Wallensten, J. Waldenstrom, A.D. Osterhaus, and R.A. Fouchier. 2006. Global patterns of influenza A virus in wild birds. *Science* 312:384-388.
- Olsen, S.J., K. Ungchusak, L. Sovann, T.M. Uyeki, S.F. Dowell, N.J. Cox, W. Aldis, and S. Chunsuttiwat. 2005. Family clustering of avian influenza A (H5N1). *Emerging infectious diseases* 11:1799-1801.
- Onodera, T., Y. Takahashi, Y. Yokoi, M. Ato, Y. Kodama, S. Hachimura, T. Kurosaki, and K. Kobayashi. 2012. Memory B cells in the lung participate in protective humoral immune responses to pulmonary influenza virus reinfection. *Proceedings of the National Academy of Sciences of the United States of America* 109:2485-2490.
- Onomoto, K., M. Jogi, J.S. Yoo, R. Narita, S. Morimoto, A. Takemura, S. Sambhara, A. Kawaguchi, S. Osari, K. Nagata, T. Matsumiya, H. Namiki, M. Yoneyama, and T. Fujita. 2012. Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity. *PloS one* 7:e43031.
- Organization, W.H. 2015. Influenza. In.
- Oshiumi, H., M. Miyashita, N. Inoue, M. Okabe, M. Matsumoto, and T. Seya. 2010. The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell host & microbe* 8:496-509.
- Osuka, F., Y. Endo, M. Higuchi, H. Suzuki, Y. Shio, K. Fujiu, R. Kanno, A. Oishi, M. Terashima, T. Fujita, and M. Gotoh. 2006. Molecular cloning and characterization of novel splicing variants of human decay-accelerating factor. *Genomics* 88:316-322.
- Palese, P. 2004. Influenza: old and new threats. *Nature medicine* 10:S82-87.
- Pang, I.K., and A. Iwasaki. 2011. Inflammasomes as mediators of immunity against influenza virus. *Trends in immunology* 32:34-41.
- Pang, I.K., P.S. Pillai, and A. Iwasaki. 2013. Efficient influenza A virus replication in the respiratory tract requires signals from TLR7 and RIG-I. *Proceedings of the National Academy of Sciences of the United States of America* 110:13910-13915.

- Parlato, S., S.M. Santini, C. Lapenta, T. Di Pucchio, M. Logozzi, M. Spada, A.M. Giammarioli, W. Malorni, S. Fais, and F. Belardelli. 2001. Expression of CCR-7, MIP-3beta, and Th-1 chemokines in type I IFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities. *Blood* 98:3022-3029.
- Pavlovic, J., H.A. Arzet, H.P. Hefti, M. Frese, D. Rost, B. Ernst, E. Kolb, P. Staeheli, and O. Haller. 1995. Enhanced virus resistance of transgenic mice expressing the human MxA protein. *Journal of virology* 69:4506-4510.
- Pavlovic, J., O. Haller, and P. Staeheli. 1992. Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *Journal of virology* 66:2564-2569.
- Peiris, J.S., W.C. Yu, C.W. Leung, C.Y. Cheung, W.F. Ng, J.M. Nicholls, T.K. Ng, K.H. Chan, S.T. Lai, W.L. Lim, K.Y. Yuen, and Y. Guan. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 363:617-619.
- Pelletier, A.N., F. Guimont-Desrochers, M.P. Ashton, T.C. Brodnicki, and S. Lesage. 2012. The size of the plasmacytoid dendritic cell compartment is a multigenic trait dominated by a locus on mouse chromosome 7. *Journal of immunology* 188:5561-5570.
- Peper, R.L., and H. Van Campen. 1995. Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia. *Microbial pathogenesis* 19:175-183.
- Perez-Padilla, R., R. Fernandez, C. Garcia-Sancho, F. Franco-Marina, O. Aburto, H. Lopez-Gatell, and I. Bojorquez. 2010. Pandemic (H1N1) 2009 virus and Down syndrome patients. *Emerging infectious diseases* 16:1312-1314.
- Perrone, L.A., J.K. Plowden, A. Garcia-Sastre, J.M. Katz, and T.M. Tumpey. 2008. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS pathogens* 4:e1000115.
- Pestka, S., C.D. Krause, and M.R. Walter. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunological reviews* 202:8-32.
- Pichlmair, A., O. Schulz, C.P. Tan, T.I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314:997-1001.
- Piehl, J., L.C. Roisman, and G. Schreiber. 2000. New structural and functional aspects of the type I interferon-receptor interaction revealed by comprehensive mutational analysis of the binding interface. *The Journal of biological chemistry* 275:40425-40433.
- Pinto, L.H., L.J. Holsinger, and R.A. Lamb. 1992. Influenza virus M2 protein has ion channel activity. *Cell* 69:517-528.
- Pinto, L.H., and R.A. Lamb. 2006. Influenza virus proton channels. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology* 5:629-632.
- Pitzer, V.E., S.J. Olsen, C.T. Bergstrom, S.F. Dowell, and M. Lipsitch. 2007. Little evidence for genetic susceptibility to influenza A (H5N1) from family clustering data. *Emerging infectious diseases* 13:1074-1076.
- Platanias, L.C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nature reviews. Immunology* 5:375-386.
- Poehling, K.A., K.M. Edwards, G.A. Weinberg, P. Szilagyi, M.A. Staat, M.K. Iwane, C.B. Bridges, C.G. Grijalva, Y. Zhu, D.I. Bernstein, G. Herrera, D. Erdman, C.B. Hall,

- R. Seither, M.R. Griffin, and N. New Vaccine Surveillance. 2006. The underrecognized burden of influenza in young children. *The New England journal of medicine* 355:31-40.
- Price, G.E., A. Gaszewska-Mastarlarz, and D. Moskophidis. 2000. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. *Journal of virology* 74:3996-4003.
- Prokunina-Olsson, L., B. Muchmore, W. Tang, R.M. Pfeiffer, H. Park, H. Dickensheets, D. Hergott, P. Porter-Gill, A. Mumy, I. Kohaar, S. Chen, N. Brand, M. Tarway, L. Liu, F. Sheikh, J. Astemborski, H.L. Bonkovsky, B.R. Edlin, C.D. Howell, T.R. Morgan, D.L. Thomas, B. Rehmann, R.P. Donnelly, and T.R. O'Brien. 2013. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nature genetics* 45:164-171.
- Puttur, F., C. Arnold-Schrauf, K. Lahl, G. Solmaz, M. Lindenberg, C.T. Mayer, M. Gohmert, M. Swallow, C. van Helt, H. Schmitt, L. Nitschke, B.N. Lambrecht, R. Lang, M. Messerle, and T. Sparwasser. 2013. Absence of Siglec-H in MCMV infection elevates interferon alpha production but does not enhance viral clearance. *PLoS pathogens* 9:e1003648.
- Qian, J., H. Zheng, W.C. Huangfu, J. Liu, C.J. Carbone, N.A. Leu, D.P. Baker, and S.Y. Fuchs. 2011. Pathogen recognition receptor signaling accelerates phosphorylation-dependent degradation of IFNAR1. *PLoS pathogens* 7:e1002065.
- Radigan, K.A., A.V. Misharin, M. Chi, and G.S. Budinger. 2015. Modeling human influenza infection in the laboratory. *Infection and drug resistance* 8:311-320.
- Radigan, K.A., D. Urich, A.V. Misharin, S.E. Chiarella, S. Soberanes, A. Gonzalez, H. Perlman, R.G. Wunderink, G.R. Budinger, and G.M. Mutlu. 2012. The effect of rosvastatin in a murine model of influenza A infection. *PloS one* 7:e35788.
- Rajasuriar, R., G. Khoury, A. Kamarulzaman, M.A. French, P.U. Cameron, and S.R. Lewin. 2013. Persistent immune activation in chronic HIV infection: do any interventions work? *Aids* 27:1199-1208.
- Randall, R.E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *The Journal of general virology* 89:1-47.
- Rehwinkel, J., C.P. Tan, D. Goubau, O. Schulz, A. Pichlmair, K. Bier, N. Robb, F. Vreede, W. Barclay, E. Fodor, and C. Reis e Sousa. 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* 140:397-408.
- Rimmelzwaan, G.F., A.C. Boon, J.T. Voeten, E.G. Berkhoff, R.A. Fouchier, and A.D. Osterhaus. 2004. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus research* 103:97-100.
- Robinson, J.L., B.E. Lee, J. Patel, N. Bastien, K. Grimsrud, R.F. Seal, R. King, F. Marshall, and Y. Li. 2007. Swine influenza (H3N2) infection in a child and possible community transmission, Canada. *Emerging infectious diseases* 13:1865-1870.
- Rodgers, B.C., and C.A. Mims. 1982. Influenza virus replication in human alveolar macrophages. *Journal of medical virology* 9:177-184.

- Roisman, L.C., D.A. Jaitin, D.P. Baker, and G. Schreiber. 2005. Mutational analysis of the IFNAR1 binding site on IFNalpha2 reveals the architecture of a weak ligand-receptor binding-site. *Journal of molecular biology* 353:271-281.
- Roman, E., E. Miller, A. Harmsen, J. Wiley, U.H. Von Andrian, G. Huston, and S.L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *The Journal of experimental medicine* 196:957-968.
- Ronnblom, L., U. Ramstedt, and G.V. Alm. 1983. Properties of human natural interferon-producing cells stimulated by tumor cell lines. *European journal of immunology* 13:471-476.
- Rothenberg, M.E., R. Ownbey, P.D. Mehlhop, P.M. Loiselle, M. van de Rijn, J.V. Bonventre, H.C. Oettgen, P. Leder, and A.D. Luster. 1996. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice. *Molecular medicine* 2:334-348.
- Sadler, A.J., and B.R. Williams. 2008. Interferon-inducible antiviral effectors. *Nature reviews. Immunology* 8:559-568.
- Safronetz, D., B. Rockx, F. Feldmann, S.E. Belisle, R.E. Palermo, D. Brining, D. Gardner, S.C. Proll, A. Marzi, Y. Tsuda, R.A. Lacasse, L. Kercher, A. York, M.J. Korth, D. Long, R. Rosenke, W.L. Shupert, C.A. Aranda, J.S. Mattoon, D. Kobasa, G. Kobinger, Y. Li, J.K. Taubenberger, J.A. Richt, M. Parnell, H. Ebihara, Y. Kawaoka, M.G. Katze, and H. Feldmann. 2011. Pandemic swine-origin H1N1 influenza A virus isolates show heterogeneous virulence in macaques. *Journal of virology* 85:1214-1223.
- Sakabe, S., K. Iwatsuki-Horimoto, R. Takano, C.A. Nidom, M. Le, T. Nagamura-Inoue, T. Horimoto, N. Yamashita, and Y. Kawaoka. 2011. Cytokine production by primary human macrophages infected with highly pathogenic H5N1 or pandemic H1N1 2009 influenza viruses. *The Journal of general virology* 92:1428-1434.
- Salomon, R., P. Staeheli, G. Kochs, H.L. Yen, J. Franks, J.E. Rehg, R.G. Webster, and E. Hoffmann. 2007. Mx1 gene protects mice against the highly lethal human H5N1 influenza virus. *Cell cycle* 6:2417-2421.
- Samji, T. 2009. Influenza A: understanding the viral life cycle. *The Yale journal of biology and medicine* 82:153-159.
- Sandberg, K., M.L. Eloranta, A. Johannisson, and G.V. Alm. 1991. Flow cytometric analysis of natural interferon-alpha producing cells. *Scandinavian journal of immunology* 34:565-576.
- Sandbulte, M.R., A.C. Boon, R.J. Webby, and J.M. Riberdy. 2008. Analysis of cytokine secretion from human plasmacytoid dendritic cells infected with H5N1 or low-pathogenicity influenza viruses. *Virology* 381:22-28.
- Sandler, N.G., S.E. Bosinger, J.D. Estes, R.T. Zhu, G.K. Tharp, E. Boritz, D. Levin, S. Wijeyesinghe, K.N. Makamdop, G.Q. del Prete, B.J. Hill, J.K. Timmer, E. Reiss, G. Yarden, S. Darko, E. Contijoch, J.P. Todd, G. Silvestri, M. Nason, R.B. Norgren, Jr., B.F. Keele, S. Rao, J.A. Langer, J.D. Lifson, G. Schreiber, and D.C. Douek. 2014. Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. *Nature* 511:601-605.
- Santini, S.M., C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. 2000. Type I interferon as a powerful adjuvant for monocyte-

- derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *The Journal of experimental medicine* 191:1777-1788.
- Santodonato, L., G. D'Agostino, R. Nisini, S. Mariotti, D.M. Monque, M. Spada, L. Lattanzi, M.P. Perrone, M. Andreotti, F. Belardelli, and M. Ferrantini. 2003. Monocyte-derived dendritic cells generated after a short-term culture with IFN-alpha and granulocyte-macrophage colony-stimulating factor stimulate a potent Epstein-Barr virus-specific CD8+ T cell response. *Journal of immunology* 170:5195-5202.
- Schaefer, U., O. Voloshanenko, D. Willen, and H. Walczak. 2007. TRAIL: a multifunctional cytokine. *Frontiers in bioscience : a journal and virtual library* 12:3813-3824.
- Scheller, J., A. Chalaris, D. Schmidt-Arras, and S. Rose-John. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et biophysica acta* 1813:878-888.
- Scherle, P.A., G. Palladino, and W. Gerhard. 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *Journal of immunology* 148:212-217.
- Schlaak, J.F., C.M. Hilken, A.P. Costa-Pereira, B. Strobl, F. Aberger, A.M. Frischauf, and I.M. Kerr. 2002. Cell-type and donor-specific transcriptional responses to interferon-alpha. Use of customized gene arrays. *The Journal of biological chemistry* 277:49428-49437.
- Schliehe, C., E.K. Flynn, B. Vilagos, U. Richson, S. Swaminathan, B. Bosnjak, L. Bauer, R.K. Kandasamy, I.M. Griesshammer, L. Kosack, F. Schmitz, V. Litvak, J. Sissons, A. Lercher, A. Bhattacharya, K. Khamina, A.L. Trivett, L. Tessarollo, I. Mesteri, A. Hladik, D. Merkler, S. Kubicek, S. Knapp, M.M. Epstein, D.E. Symer, A. Aderem, and A. Bergthaler. 2015. The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection. *Nature immunology* 16:67-74.
- Schmitz, N., M. Kurrer, M.F. Bachmann, and M. Kopf. 2005. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *Journal of virology* 79:6441-6448.
- Schneider, C., S.P. Nobs, A.K. Heer, M. Kurrer, G. Klinke, N. van Rooijen, J. Vogel, and M. Kopf. 2014a. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS pathogens* 10:e1004053.
- Schneider, W.M., M.D. Chevillotte, and C.M. Rice. 2014b. Interferon-stimulated genes: a complex web of host defenses. *Annual review of immunology* 32:513-545.
- Schroder, K., P.J. Hertzog, T. Ravasi, and D.A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology* 75:163-189.
- Schulz, O., S.S. Diebold, M. Chen, T.I. Naslund, M.A. Nolte, L. Alexopoulou, Y.T. Azuma, R.A. Flavell, P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433:887-892.
- Schusser, B., A. Reuter, A. von der Malsburg, N. Penski, S. Weigend, B. Kaspers, P. Staeheli, and S. Hartle. 2011. Mx is dispensable for interferon-mediated resistance of chicken cells against influenza A virus. *Journal of virology* 85:8307-8315.

- Selikowitz, M. 1992. Health problems and health checks in school-aged children with Down syndrome. *Journal of paediatrics and child health* 28:383-386.
- Seo, S.U., H.J. Kwon, H.J. Ko, Y.H. Byun, B.L. Seong, S. Uematsu, S. Akira, and M.N. Kweon. 2011. Type I interferon signaling regulates Ly6C(hi) monocytes and neutrophils during acute viral pneumonia in mice. *PLoS pathogens* 7:e1001304.
- Serbina, N.V., and E.G. Pamer. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature immunology* 7:311-317.
- Shahangian, A., E.K. Chow, X. Tian, J.R. Kang, A. Ghaffari, S.Y. Liu, J.A. Belperio, G. Cheng, and J.C. Deng. 2009. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *The Journal of clinical investigation* 119:1910-1920.
- Shaw, S., and W.E. Biddison. 1979. HLA-linked genetic control of the specificity of human cytotoxic T-cell responses to influenza virus. *The Journal of experimental medicine* 149:565-575.
- Sheppard, O., F.K. Wiseman, A. Ruparel, V.L. Tybulewicz, and E.M. Fisher. 2012. Mouse models of aneuploidy. *TheScientificWorldJournal* 2012:214078.
- Sheppard, P., W. Kindsvogel, W. Xu, K. Henderson, S. Schlutsmeyer, T.E. Whitmore, R. Kuestner, U. Garrigues, C. Birks, J. Roraback, C. Ostrander, D. Dong, J. Shin, S. Presnell, B. Fox, B. Haldeman, E. Cooper, D. Taft, T. Gilbert, F.J. Grant, M. Tackett, W. Krivan, G. McKnight, C. Clegg, D. Foster, and K.M. Klucher. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nature immunology* 4:63-68.
- Shin, D.L., B. Hatesuer, S. Bergmann, T. Nedelko, and K. Schughart. 2015. Protection from Severe Influenza Virus Infections in Mice Carrying the Mx1 Influenza Virus Resistance Gene Strongly Depends on Genetic Background. *Journal of virology* 89:9998-10009.
- Shiow, L.R., D.B. Rosen, N. Brdickova, Y. Xu, J. An, L.L. Lanier, J.G. Cyster, and M. Matloubian. 2006. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 440:540-544.
- Shukla, P., G.M. Rao, G. Pandey, S. Sharma, N. Mittapelly, R. Shegokar, and P.R. Mishra. 2014. Therapeutic interventions in sepsis: current and anticipated pharmacological agents. *British journal of pharmacology* 171:5011-5031.
- Si, Y., C.L. Tsou, K. Croft, and I.F. Charo. 2010. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *The Journal of clinical investigation* 120:1192-1203.
- Siegal, F.P., N. Kadowaki, M. Shodell, P.A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y.J. Liu. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 284:1835-1837.
- Simonsen, L., P. Spreeuwenberg, R. Lustig, R.J. Taylor, D.M. Fleming, M. Kroneman, M.D. Van Kerkhove, A.W. Mounts, W.J. Paget, and G.L.C. Teams. 2013. Global mortality estimates for the 2009 Influenza Pandemic from the GLaMOR project: a modeling study. *PLoS medicine* 10:e1001558.
- Skountzou, I., D.G. Koutsouanos, J.H. Kim, R. Powers, L. Satyabhama, F. Maseoud, W.C. Weldon, P. Martin Mdel, R.S. Mittler, R. Compans, and J. Jacob. 2010. Immunity to pre-1950 H1N1 influenza viruses confers cross-protection

- against the pandemic swine-origin 2009 A (H1N1) influenza virus. *Journal of immunology* 185:1642-1649.
- Smed-Sorensen, A., C. Chalouni, B. Chatterjee, L. Cohn, P. Blattmann, N. Nakamura, L. Delamarre, and I. Mellman. 2012. Influenza A virus infection of human primary dendritic cells impairs their ability to cross-present antigen to CD8 T cells. *PLoS pathogens* 8:e1002572.
- Smith, W., Andrews, C.H. and Laidlaw. P.P. 1933. A virus obtained from influenza patients. *Lancet* 66-68.
- Snelgrove, R.J., L. Edwards, A.J. Rae, and T. Hussell. 2006. An absence of reactive oxygen species improves the resolution of lung influenza infection. *European journal of immunology* 36:1364-1373.
- Soloff, A.C., H.K. Weirback, T.M. Ross, and S.M. Barratt-Boyes. 2012. Plasmacytoid dendritic cell depletion leads to an enhanced mononuclear phagocyte response in lungs of mice with lethal influenza virus infection. *Comparative immunology, microbiology and infectious diseases* 35:309-317.
- Sommerey, C., S. Paul, P. Staeheli, and T. Michiels. 2008. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS pathogens* 4:e1000017.
- Soumelis, V., I. Scott, F. Gheyas, D. Bouhour, G. Cozon, L. Cotte, L. Huang, J.A. Levy, and Y.J. Liu. 2001. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood* 98:906-912.
- Sprenger, H., R.G. Meyer, A. Kaufmann, D. Bussfeld, E. Rischkowsky, and D. Gems. 1996. Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. *The Journal of experimental medicine* 184:1191-1196.
- Srivastava, B., P. Blazejewski, M. Hessmann, D. Bruder, R. Geffers, S. Maue, A.D. Gruber, and K. Schughart. 2009. Host genetic background strongly influences the response to influenza A virus infections. *PloS one* 4:e4857.
- Staeheli, P., P. Dreiding, O. Haller, and J. Lindenmann. 1985. Polyclonal and monoclonal antibodies to the interferon-inducible protein Mx of influenza virus-resistant mice. *The Journal of biological chemistry* 260:1821-1825.
- Starr, S.E., S. Bandyopadhyay, V. Shanmugam, N. Hassan, S. Douglas, S.J. Jackson, G. Trinchieri, and J. Chehimi. 1993. Morphological and functional differences between HLA-DR+ peripheral blood dendritic cells and HLA-DR+ IFN-alpha producing cells. *Advances in experimental medicine and biology* 329:173-178.
- Stary, G., I. Klein, S. Kohlhofer, F. Koszik, T. Scherzer, L. Mullauer, H. Quendler, N. Kohrgruber, and G. Stingl. 2009. Plasmacytoid dendritic cells express TRAIL and induce CD4+ T-cell apoptosis in HIV-1 viremic patients. *Blood* 114:3854-3863.
- Steidle, S., L. Martinez-Sobrido, M. Mordstein, S. Lienenklaus, A. Garcia-Sastre, P. Staeheli, and G. Kochs. 2010. Glycine 184 in nonstructural protein NS1 determines the virulence of influenza A virus strain PR8 without affecting the host interferon response. *Journal of virology* 84:12761-12770.
- Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *The EMBO journal* 11:2407-2414.
- Stohr, K. 2002. Influenza--WHO cares. *The Lancet. Infectious diseases* 2:517.

- Strasser, A., P.J. Jost, and S. Nagata. 2009. The many roles of FAS receptor signaling in the immune system. *Immunity* 30:180-192.
- Sui, J., W.C. Hwang, S. Perez, G. Wei, D. Aird, L.M. Chen, E. Santelli, B. Stec, G. Cadwell, M. Ali, H. Wan, A. Murakami, A. Yammanuru, T. Han, N.J. Cox, L.A. Bankston, R.O. Donis, R.C. Liddington, and W.A. Marasco. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature structural & molecular biology* 16:265-273.
- Sun, J., R. Madan, C.L. Karp, and T.J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nature medicine* 15:277-284.
- Swiecki, M., Y. Wang, W. Vermi, S. Gilfillan, R.D. Schreiber, and M. Colonna. 2011. Type I interferon negatively controls plasmacytoid dendritic cell numbers in vivo. *The Journal of experimental medicine* 208:2367-2374.
- Szretter, K.J., S. Gangappa, J.A. Belser, H. Zeng, H. Chen, Y. Matsuoka, S. Sambhara, D.E. Swayne, T.M. Tumpey, and J.M. Katz. 2009. Early control of H5N1 influenza virus replication by the type I interferon response in mice. *Journal of virology* 83:5825-5834.
- Szretter, K.J., S. Gangappa, X. Lu, C. Smith, W.J. Shieh, S.R. Zaki, S. Sambhara, T.M. Tumpey, and J.M. Katz. 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. *Journal of virology* 81:2736-2744.
- Tai, L.H., M.L. Goulet, S. Belanger, N. Toyama-Sorimachi, N. Fodil-Cornu, S.M. Vidal, A.D. Troke, D.W. McVicar, and A.P. Makrigiannis. 2008. Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *The Journal of experimental medicine* 205:3187-3199.
- Talon, J., C.M. Horvath, R. Polley, C.F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre. 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *Journal of virology* 74:7989-7996.
- Tan, G.S., F. Krammer, D. Eggink, A. Kongchanagul, T.M. Moran, and P. Palese. 2012. A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. *Journal of virology* 86:6179-6188.
- Tan, G.S., P.S. Lee, R.M. Hoffman, B. Mazel-Sanchez, F. Krammer, P.E. Leon, A.B. Ward, I.A. Wilson, and P. Palese. 2014. Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. *Journal of virology* 88:13580-13592.
- Taubenberger, J.K., and D.M. Morens. 2006. 1918 Influenza: the mother of all pandemics. *Emerging infectious diseases* 12:15-22.
- Teijaro, J.R., C. Ng, A.M. Lee, B.M. Sullivan, K.C. Sheehan, M. Welch, R.D. Schreiber, J.C. de la Torre, and M.B. Oldstone. 2013. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 340:207-211.
- Teijaro, J.R., K.B. Walsh, S. Cahalan, D.M. Fremgen, E. Roberts, F. Scott, E. Martinborough, R. Peach, M.B. Oldstone, and H. Rosen. 2011. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. *Cell* 146:980-991.
- Terczyńska-Dyla, E.S.B., Francois H. T. Duong, Ilona Krol, Sanne Jørgensen, Emilie Collinet, Zoltán Kutalik, Vincent Aubert, Andreas Cerny, Laurent Kaiser, Raffaele Malinverni, Alessandra Mangia, Darius Moradpour, Beat Müllhaupt, Francesco Negro, Rosanna Santoro, David Semela, Nasser

- Semmo, Swiss Hepatitis C Cohort Study Group, Markus H. Heim, Pierre-Yves Bochud & Rune Hartmann . 2015. Reduced IFN λ 4 activity is associated with improved HCV clearance and reduced expression of interferon-stimulated genes. *Nature communications* 5:
- Thitithanyanont, A., A. Engering, P. Ekchariyawat, S. Wiboon-ut, A. Limsalakpetch, K. Yongvanitchit, U. Kum-Arb, W. Kanchongkittiphon, P. Utaisincharoen, S. Sirisinha, P. Puthavathana, M.M. Fukuda, and S. Pichyangkul. 2007. High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. *Journal of immunology* 179:5220-5227.
- Thomas, P.G., P. Dash, J.R. Aldridge, Jr., A.H. Ellebedy, C. Reynolds, A.J. Funk, W.J. Martin, M. Lamkanfi, R.J. Webby, K.L. Boyd, P.C. Doherty, and T.D. Kanneganti. 2009. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30:566-575.
- Threadgill, D.W., and G.A. Churchill. 2012. Ten years of the collaborative cross. *G3* 2:153-156.
- Tilg, H., J.W. Mier, W. Vogel, W.E. Aulitzky, C.J. Wiedermann, E. Vannier, C. Huber, and C.A. Dinarello. 1993. Induction of circulating IL-1 receptor antagonist by IFN treatment. *Journal of immunology* 150:4687-4692.
- Tinel, N., S. Diochot, M. Borsotto, M. Lazdunski, and J. Barhanin. 2000. KCNE2 confers background current characteristics to the cardiac KCNQ1 potassium channel. *The EMBO journal* 19:6326-6330.
- Topham, D.J., R.A. Tripp, and P.C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *Journal of immunology* 159:5197-5200.
- Topham, D.J., R.A. Tripp, A.M. Hamilton-Easton, S.R. Sarawar, and P.C. Doherty. 1996. Quantitative analysis of the influenza virus-specific CD4+ T cell memory in the absence of B cells and Ig. *Journal of immunology* 157:2947-2952.
- Toth, L.A., and R.W. Williams. 1999. A quantitative genetic analysis of slow-wave sleep in influenza-infected CXB recombinant inbred mice. *Behavior genetics* 29:339-348.
- Townsend, A.R., F.M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* 42:457-467.
- Trammell, R.A., T.A. Liberati, and L.A. Toth. 2012. Host genetic background and the innate inflammatory response of lung to influenza virus. *Microbes and infection / Institut Pasteur* 14:50-58.
- Trapnell, B.C., and J.A. Whitsett. 2002. Gm-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. *Annual review of physiology* 64:775-802.
- Treanor, J.J., E.L. Tierney, S.L. Zebedee, R.A. Lamb, and B.R. Murphy. 1990. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *Journal of virology* 64:1375-1377.
- Trinchieri, G. 2010. Type I interferon: friend or foe? *The Journal of experimental medicine* 207:2053-2063.
- Tumpey, T.M., C.F. Basler, P.V. Aguilar, H. Zeng, A. Solorzano, D.E. Swayne, N.J. Cox, J.M. Katz, J.K. Taubenberger, P. Palese, and A. Garcia-Sastre. 2005a.

- Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310:77-80.
- Tumpey, T.M., A. Garcia-Sastre, J.K. Taubenberger, P. Palese, D.E. Swayne, M.J. Pantin-Jackwood, S. Schultz-Cherry, A. Solorzano, N. Van Rooijen, J.M. Katz, and C.F. Basler. 2005b. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *Journal of virology* 79:14933-14944.
- Tumpey, T.M., K.J. Szretter, N. Van Hoeven, J.M. Katz, G. Kochs, O. Haller, A. Garcia-Sastre, and P. Staeheli. 2007. The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. *Journal of virology* 81:10818-10821.
- Turner, S., P. Sloper, C. Cunningham, and C. Knussen. 1990. Health problems in children with Down's syndrome. *Child: care, health and development* 16:83-97.
- Tyner, J.W., O. Uchida, N. Kajiwar, E.Y. Kim, A.C. Patel, M.P. O'Sullivan, M.J. Walter, R.A. Schwendener, D.N. Cook, T.M. Danoff, and M.J. Holtzman. 2005. CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection. *Nature medicine* 11:1180-1187.
- Van Hoeven, N., J.A. Belser, K.J. Szretter, H. Zeng, P. Staeheli, D.E. Swayne, J.M. Katz, and T.M. Tumpey. 2009. Pathogenesis of 1918 pandemic and H5N1 influenza virus infections in a guinea pig model: antiviral potential of exogenous alpha interferon to reduce virus shedding. *Journal of virology* 83:2851-2861.
- van Riel, D., L.M. Leijten, M. van der Eerden, H.C. Hoogsteden, L.A. Boven, B.N. Lambrecht, A.D. Osterhaus, and T. Kuiken. 2011. Highly pathogenic avian influenza virus H5N1 infects alveolar macrophages without virus production or excessive TNF-alpha induction. *PLoS pathogens* 7:e1002099.
- van Riel, D., V.J. Munster, E. de Wit, G.F. Rimmelzwaan, R.A. Fouchier, A.D. Osterhaus, and T. Kuiken. 2006. H5N1 Virus Attachment to Lower Respiratory Tract. *Science* 312:399.
- van Riel, D., V.J. Munster, E. de Wit, G.F. Rimmelzwaan, R.A. Fouchier, A.D. Osterhaus, and T. Kuiken. 2007. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *The American journal of pathology* 171:1215-1223.
- Verhelst, J., E. Parthoens, B. Schepens, W. Fiers, and X. Saelens. 2012. Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly. *Journal of virology* 86:13445-13455.
- Verstegen, R.H., M.A. Kusters, E.F. Gemen, and D.E.V. E. 2010. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. *Pediatric research* 67:563-569.
- Voeten, J.T., T.M. Bestebroer, N.J. Nieuwkoop, R.A. Fouchier, A.D. Osterhaus, and G.F. Rimmelzwaan. 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *Journal of virology* 74:6800-6807.
- Walczak, H., and T.L. Haas. 2008. Biochemical analysis of the native TRAIL death-inducing signaling complex. *Methods in molecular biology* 414:221-239.

- Wang, M., K. Lamberth, M. Harndahl, G. Roder, A. Stryhn, M.V. Larsen, M. Nielsen, C. Lundegaard, S.T. Tang, M.H. Dziegiel, J. Rosenkvist, A.E. Pedersen, S. Buus, M.H. Claesson, and O. Lund. 2007. CTL epitopes for influenza A including the H5N1 bird flu; genome-, pathogen-, and HLA-wide screening. *Vaccine* 25:2823-2831.
- Wang, W., P. Yang, Y. Zhong, Z. Zhao, L. Xing, Y. Zhao, Z. Zou, Y. Zhang, C. Li, T. Li, C. Wang, Z. Wang, X. Yu, B. Cao, X. Gao, J.M. Penninger, X. Wang, and C. Jiang. 2013. Monoclonal antibody against CXCL-10/IP-10 ameliorates influenza A (H1N1) virus induced acute lung injury. *Cell research* 23:577-580.
- Wang, X., M. Li, H. Zheng, T. Muster, P. Palese, A.A. Beg, and A. Garcia-Sastre. 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *Journal of virology* 74:11566-11573.
- Wang, Z., A. Zhang, Y. Wan, X. Liu, C. Qiu, X. Xi, Y. Ren, J. Wang, Y. Dong, M. Bao, L. Li, M. Zhou, S. Yuan, J. Sun, Z. Zhu, L. Chen, Q. Li, Z. Zhang, X. Zhang, S. Lu, P.C. Doherty, K. Kedzierska, and J. Xu. 2014. Early hypercytokinemia is associated with interferon-induced transmembrane protein-3 dysfunction and predictive of fatal H7N9 infection. *Proceedings of the National Academy of Sciences of the United States of America* 111:769-774.
- Ward, C.L., M.H. Dempsey, C.J. Ring, R.E. Kempson, L. Zhang, D. Gor, B.W. Snowden, and M. Tisdale. 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 29:179-188.
- Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiological reviews* 56:152-179.
- Weidner, J.M., D. Jiang, X.B. Pan, J. Chang, T.M. Block, and J.T. Guo. 2010. Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. *Journal of virology* 84:12646-12657.
- Wetzel, J.L., V. Fensterl, and G.C. Sen. 2014. Sendai virus pathogenesis in mice is prevented by Ifit2 and exacerbated by interferon. *Journal of virology* 88:13593-13601.
- Wijburg, O.L., S. DiNatale, J. Vadolas, N. van Rooijen, and R.A. Strugnell. 1997. Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. *Journal of virology* 71:9450-9457.
- Wilson, E.B., D.H. Yamada, H. Elsaesser, J. Herskovitz, J. Deng, G. Cheng, B.J. Aronow, C.L. Karp, and D.G. Brooks. 2013. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* 340:202-207.
- Wilson, I.A., J.J. Skehel, and D.C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289:366-373.
- Wolf, A.I., D. Buehler, S.E. Hensley, L.L. Cavanagh, E.J. Wherry, P. Kastner, S. Chan, and W. Weninger. 2009. Plasmacytoid dendritic cells are dispensable during primary influenza virus infection. *Journal of immunology* 182:871-879.
- Woo, P.C., E.T. Tung, K.H. Chan, C.C. Lau, S.K. Lau, and K.Y. Yuen. 2010. Cytokine profiles induced by the novel swine-origin influenza A/H1N1 virus:

- implications for treatment strategies. *The Journal of infectious diseases* 201:346-353.
- Yamaji, K., S. Nabeshima, M. Murata, Y. Chong, N. Furusyo, H. Ikematsu, and J. Hayashi. 2006. Interferon-alpha/beta upregulate IL-15 expression in vitro and in vivo: analysis in human hepatocellular carcinoma cell lines and in chronic hepatitis C patients during interferon-alpha/beta treatment. *Cancer immunology, immunotherapy: CII* 55:394-403.
- Yamazaki, Y., M. Yamada, T. Kawai, T. Morio, M. Onodera, M. Ueki, N. Watanabe, H. Takada, S. Takezaki, N. Chida, I. Kobayashi, and T. Ariga. 2014. Two novel gain-of-function mutations of STAT1 responsible for chronic mucocutaneous candidiasis disease: impaired production of IL-17A and IL-22, and the presence of anti-IL-17F autoantibody. *Journal of immunology* 193:4880-4887.
- Yang, Y.T., and C.A. Evans. 1961. Hypothermia in mice due to influenza virus infection. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* 108:776-780.
- Yewdell, J.W., J.R. Bennink, G.L. Smith, and B. Moss. 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 82:1785-1789.
- Yoshida, R., H.W. Murray, and C.F. Nathan. 1988. Agonist and antagonist effects of interferon alpha and beta on activation of human macrophages. Two classes of interferon gamma receptors and blockade of the high-affinity sites by interferon alpha or beta. *The Journal of experimental medicine* 167:1171-1185.
- Youle, R.J., and A. Strasser. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews. Molecular cell biology* 9:47-59.
- Yu, W.C., R.W. Chan, J. Wang, E.A. Travanty, J.M. Nicholls, J.S. Peiris, R.J. Mason, and M.C. Chan. 2011. Viral replication and innate host responses in primary human alveolar epithelial cells and alveolar macrophages infected with influenza H5N1 and H1N1 viruses. *Journal of virology* 85:6844-6855.
- Zebedee, S.L., and R.A. Lamb. 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *Journal of virology* 62:2762-2772.
- Zeng, H., C. Goldsmith, P. Thawatsupha, M. Chittaganpitch, S. Waicharoen, S. Zaki, T.M. Tumpey, and J.M. Katz. 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type I interferon response in polarized human bronchial epithelial cells. *Journal of virology* 81:12439-12449.
- Zhang, L., S. Yuan, G. Cheng, and B. Guo. 2011. Type I IFN promotes IL-10 production from T cells to suppress Th17 cells and Th17-associated autoimmune inflammation. *PloS one* 6:e28432.
- Zhang, S.Y., S. Boisson-Dupuis, A. Chapgier, K. Yang, J. Bustamante, A. Puel, C. Picard, L. Abel, E. Jouanguy, and J.L. Casanova. 2008. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. *Immunological reviews* 226:29-40.
- Zhang, Y.H., Y. Zhao, N. Li, Y.C. Peng, E. Giannoulatou, R.H. Jin, H.P. Yan, H. Wu, J.H. Liu, N. Liu, D.Y. Wang, Y.L. Shu, L.P. Ho, P. Kellam, A. McMichael, and T. Dong. 2013. Interferon-induced transmembrane protein-3 genetic variant

rs12252-C is associated with severe influenza in Chinese individuals. *Nature communications* 4:1418.

- Zheng, H., J. Qian, D.P. Baker, and S.Y. Fuchs. 2011. Tyrosine phosphorylation of protein kinase D2 mediates ligand-inducible elimination of the Type 1 interferon receptor. *The Journal of biological chemistry* 286:35733-35741.
- Zhou, J., H.K. Law, C.Y. Cheung, I.H. Ng, J.S. Peiris, and Y.L. Lau. 2006. Differential expression of chemokines and their receptors in adult and neonatal macrophages infected with human or avian influenza viruses. *The Journal of infectious diseases* 194:61-70.
- Zhou, J., K.K. To, H. Dong, Z.S. Cheng, C.C. Lau, V.K. Poon, Y.H. Fan, Y.Q. Song, H. Tse, K.H. Chan, B.J. Zheng, G.P. Zhao, and K.Y. Yuen. 2012. A functional variation in CD55 increases the severity of 2009 pandemic H1N1 influenza A virus infection. *The Journal of infectious diseases* 206:495-503.
- Zuniga, J., I. Buendia-Roldan, Y. Zhao, L. Jimenez, D. Torres, J. Romo, G. Ramirez, A. Cruz, G. Vargas-Alarcon, C.C. Sheu, F. Chen, L. Su, A.M. Tager, A. Pardo, M. Selman, and D.C. Christiani. 2012. Genetic variants associated with severe pneumonia in A/H1N1 influenza infection. *The European respiratory journal* 39:604-610.

Wipe away the bad and keep only the good.